Raquel Carvalho de Ferreira Costa e Almeida

Crosstalk between endothelial progenitor cells and fibroblasts in vascularization

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This work was supervised by:

Professora Doutora Raquel Soares
Faculdade de Medicina da Universidade do Porto
Departamento de Bioquímica (U38-FCT)

and

Doutor Pedro Lopes Granja
Faculdade de Engenharia da Universidade do Porto
INEB – Instituto de Engenharia Biomédica

Host institutions:

Centro de Investigação Médica
Faculdade de Medicina da Universidade do Porto
Departamento de Bioquímica (U38-FCT)
Rua Dr. Plácido da Costa, 91, 4200- Porto, PORTUGAL
www.med.up.pt

IBMC – INEB Associate Laboratory
Rua do Campo Alegre, 823, 4150-180 Porto, PORTUGAL
INEB – Instituto de Engenharia Biomédica www.ineb.up.pt

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Abstract

Vascularization is a major challenge in tissue engineering. Different strategies exist, and cell-based approaches have emerged as a promising therapy to achieve successful vascularization. Co-culture systems of endothelial cells (ECs) and other supporting cells may have an important role in what refers to cellular crosstalk, namely through the production of growth factors and extracellular matrix (ECM) synthesis. Since activation and migration of fibroblasts is required for several physiological events that rely on angiogenesis, the hypothesis underlying the research reported in the present work is that human outgrowth endothelial cells (OECs) and mature ECs respond differently when co-cultured with different types of human fibroblasts, in what concerns the formation of capillary-like structures. To investigate this, OECs were isolated from human umbilical cord blood samples and characterized by immunofluorescence, western blot and imaging flow cytometry. Also, two types of human dermal fibroblasts were used (neonatal human foreskin fibroblasts – HFF-1 – or juvenile human dermal fibroblasts – HDF), being characterized in terms of the expression of podoplanin (PDPN) and transglutaminase-2 (TG2), markers of dermal fibroblasts, as well as alpha smooth muscle actin (α-SMA), a marker of fibroblast activation. Co-culture systems were established using either human umbilical vein ECs (HUVECs) or OECs with HFF-1 or HDF. Two 3D systems were compared in the present work – one in 3D without the addition of an external ECM (co-culture) and another in 3D with the addition of an external ECM (matrigel) at the beginning of the assay. Several culture time points were evaluated: 7, 14 and 21 days for the co-culture system; 24h, 48h and 7 days for the matrigel assay. The formation of capillary-like structures was assessed by immunocytochemistry against CD31 and vWF proteins. Parameters such as the number of tubular structures and branching points, length and thickness were evaluated. The presence of ECM components, such as collagen types I and IV, laminin and fibronectin, was assessed in all cell types and in co-cultures of HUVEC/HDF by immunofluorescence and western blot.

OECs expressed several endothelial markers, including CD31, VE-cadherin and vWF, as well as VEGFR2 and CD34. HFF-1 exhibited a higher expression of TG2 than that observed for HDF, while HDF expressed higher amounts of PDPN and α-SMA. Fibroblasts influenced the formation of capillary-like structures by endothelial cells. Indeed, the formation of capillary-like structures was only observed in co-cultures with HDF and not with HFF-1 fibroblasts. In addition, in the co-culture system, HUVECs formed a highly branched capillary-like network to a great extent than that observed for OECs. In the matrigel assay, HUVECs and OECs behaved similarly when co-cultured

with HDF. In terms of ECM, HUVECs were found to secrete collagen type IV, fibronectin and laminin to the extracellular media, whereas in OECs these proteins were only detected intracellularly. HDF were able to secrete all the investigated ECM components, primarily collagen I, which is lacking in HUVECs, OECs and HFF-1. In co-cultures of HUVEC/HDF, it was observed that all investigated ECM components were present, with collagen types I and IV being mainly expressed where capillary-like structures were present.

These findings suggest that HDF is a preferential cell source for enhancing vascularization, both in HUVECs and OECs. Given the already described advantages of OECs, these findings open a new field of research regarding the use of specific fibroblast populations co-cultured with OECs, as efficient partners for vascular development with tissue regeneration purposes.

Keywords: Endothelial cells, Co-culture models, Fibroblasts, Tissue Regeneration, Vascularization

Resumo

A vascularização é um dos maiores desafios em engenharia de tecidos. Existem diferentes estratégias e abordagens baseadas no uso de células emergiram como terapia promissora para se alcançar, com sucesso, vascularização. Os sistemas de co-culturas de células endoteliais (CEs) com outras células de suporte podem ter um papel importante no que se refere à comunicação celular, nomeadamente através da produção de fatores de crescimento e síntese de matriz extracelular (MEC). Dado que a ativação e migração de fibroblastos são requisitos de diversos eventos fisiológicos que dependem de processos angiogénicos, a hipótese subjacente ao presente trabalho é a possibilidade de células endoteliais progenitoras tardias (OECs) e ECs maduras responderem de forma distinta quando co-cultivadas com diferentes tipos de fibroblastos, em termos de formação de estruturas semelhantes a capilares. A fim de investigar esta hipótese, OECs foram isoladas a partir de amostras de sangue do cordão umbilical humano e caracterizadas por imunofluorescência, western blot e citometria de fluxo. Dois tipos de fibroblastos humanos da derme foram igualmente usados (fibroblastos neonatais humanos – HFF-1 – ou fibroblastos juvenis humanos – HDF), sendo caracterizados em termos da expressão de marcadores fibroblásticos da derme, incluindo podoplanina (PDPN) e transglutaminase-2 (TG2), assim como α-actina de músculo liso (α-SMA), um marcador de ativação de fibroblastos. Sistemas de co-culturas foram estabelecidos, usando tanto CEs de veia umbilical humana (HUVECs) ou OECs com HFF-1 ou HDF. Dois sistemas 3D foram comparados no presente trabalho – um sem adição de uma matriz externa (co-cultura) e outro em que se adicionou uma matriz (matrigel) no início do ensaio. Foram avaliados diversos tempos de cultura. 7, 14 e 21 dias, no sistema de co-cultura; 24, 48h e 7 dias, no caso do ensaio em matrigel. A formação de capilares foi determinada através de imunocitoquímica contra as proteínas CD31 e vWF, tendo sido avaliados parâmetros como o número de estruturas tubulares e pontos de ramificação, comprimento e diâmetro. A presença de componentes da MEC, incluindo colagénios tipos I e IV, laminina e fibronectina, foi estudada em todos os tipos de células e nas co-culturas de HUVEC/HDF por imunofluorescência e western blot.

As OECs expressaram vários marcadores endoteliais, incluindo CD31, VE-caderina e vWF, bem como VEGFR2 e CD34. Os HFF-1 exibiram uma maior expressão de TG2, enquanto os HDF expressaram PDPN e α-SMA em maiores quantidades. Os fibroblastos influenciaram a formação de estruturas-tipo capilares pelas células endoteliais. De facto, a formação de estruturas-tipo capilares foi apenas observada apenas nas co-culturas com HDF e não com HFF-1. Além disso, no sistema de co-

cultura, as HUVECs formaram uma rede-tipo capilar altamente ramificada, numa maior extensão do que aquela observada no caso das OECs. No ensaio de matrigel, HUVECs e OECs tiveram um comportamento semelhante quando co-cultivadas com HDF. No que se refere à MEC, observou-se que as HUVECs secretaram colagénio tipo IV, fibronectina e laminina para o meio extracelular, enquanto as mesmas proteínas nas OECs foram detetadas apenas intracelularmente. Os HDF também secretaram todos os componentes da MEC, principalmente colagénio tipo I, que não foi encontrado nas HUVECs, OECs e HFF-1. No sistema de co-cultura de HUVEC/HDF, observou-se que estavam presentes todos os componentes da MEC investigados, sendo os colagénios tipo I e IV maioritariamente expressos onde estruturas-tipo capilares estavam presentes.

Estes resultados sugerem os HDF como um fonte preferencial de células para melhorar a vascularização, tanto com HUVECs como com OECs. Tendo em consideração as vantagens já descritas para as OECs, os presentes resultados criam uma nova área de investigação, no que respeita ao uso de populações específicas de fibroblastos em co-cultura com OECs, enquanto parceiros eficazes para o desenvolvimento vascular com fins de regeneração de tecidos.

Palavras-chave: Células endoteliais, Modelos de co-culturas, Fibroblastos, Regeneração de Tecidos, Vascularização

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Abbreviations

CD31 – Cluster of differentiation molecule 31 (=PECAM-1)

CD34 – Cluster of differentiation molecule 34

CD144 – Cluster of differentiation molecule 144 (=VE-cadherin)

CM – Conditioned medium

DMEM – Dulbecco's Modified Eagle Medium

EC – Endothelial cell

ECM – Extracellular matrix

EGM-2 – Endothelial Growth Medium-2

EGM-2MV – Microvascular Endothelial Growth Medium-2

EPC – Endothelial progenitor cell

GFR – Growth factor reduced

HDF – Juvenile human dermal fibroblast

HFF-1 – Neonatal human foreskin fibroblast

HUVEC – Human umbilical vein endothelial cell

MMP – Matrix metalloproteinase

MNC – Mononuclear cell

OEC – Outgrowth endothelial cell

PDPN - Podoplanin

SMC – Smooth muscle cell

TG2 – Transglutaminase-2

TGF-β – Transforming growth factor beta

VEGF – Vascular endothelial growth factor

VEGFR2 – Vascular endothelial growth factor receptor 2 (=Flk-1)

vWF – von Willebrand factor

α-SMA – Alpha smooth muscle actin

CHAPTER I

INTRODUCTION

1.1. The relevance of vascularization in Tissue Engineering

The regeneration of a lost or damaged tissue function in the adult generally involves a recapitulation of developmental processes, consequently implying the replication of biological concepts and instructions expressed during the embryonic development (1). This is the main reason why regeneration of large defects in a human adult tissue does not occur naturally. The majority of reconstruction and cosmetic surgeries use autologous donor sites as a source of soft tissue, which creates a secondary wound and increases surgery and recovery times, as well as a higher risk of infection and potential loss of function, resulting in a great demand for engineered/ artificial soft tissues for clinical applications (2).

Each tissue is constituted by several cell types, resulting in a complex structure, highly organized and integrated into the body, namely through the vascular and the nervous systems. Tissue engineering has been evolving as an interdisciplinary science which intends to restore, maintain or improve tissue function (3). In general terms, tissue engineering aims for the creation of adequate tissue or even organ equivalents, which is, therefore, a highly challenging task (4). Nowadays, only *in vitro* tissue engineered products for skin and cartilage are successfully used in clinics (5), since their demands in terms of blood supply are reduced, owing to the fact that these tissues are supplied through diffusion from distant blood vessels. However, the maximum diffusion distance of nutrients and oxygen out of blood vessels is 200 μm (6) and, due to mass transfer

limitations, organs with a more complex tridimensional structure actually need an integrated vascular network to support cell survival.

Overall, the inability to engineer blood vessels *in vitro* for subsequent transplantation has been referred as the main reason associated to the limited clinical success of tissue engineering strategies (5, 7, 8). When a tissue engineered construct, including transplanted cells, faces insufficient blood supply, associate problems arise due to the lack of nutrients and oxygen transportation, as well as elimination of metabolites, resulting in functional limitations of the bioengineered tissue or even to the failure of the implanted construct. Hence, several strategies have been investigated concerning vascularization for tissue engineering applications (Fig. 1.1), like growth factor delivery, cell transplantation and the use of materials for immobilization strategies (scaffold-based approaches), which have been extensively reviewed (5, 7, 9). In fact, there have been different efforts to develop new therapies so that a faster and successful vascularization is achieved.

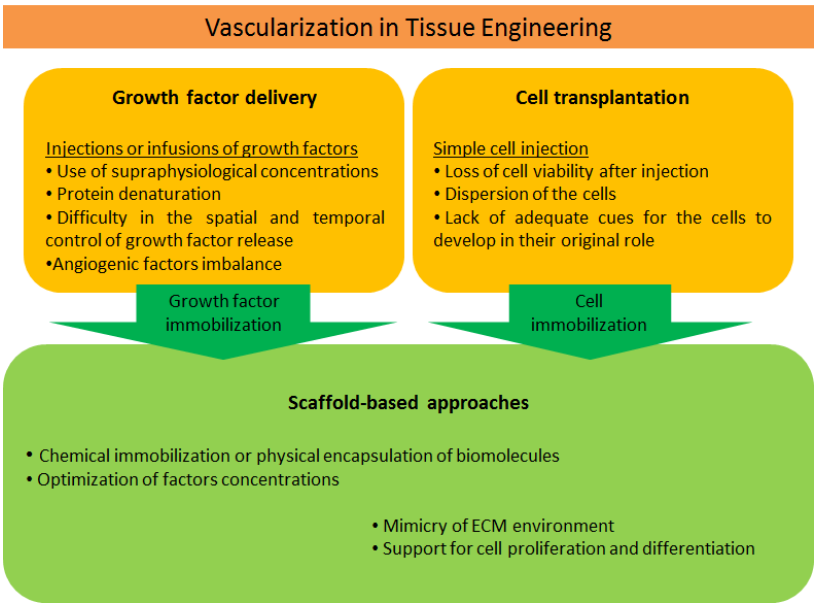


Figure 1.1. Summary of strategies used to promote vascularization for tissue engineering applications. Growth factor delivery and cell transplantation are traditional strategies that present several associated problems and have been improved through their combination and the use of scaffold materials.

1.2. The vascular network

The vascular network is responsible not only for the transport of gases, nutrients and metabolites, but also for the circulation of signalling molecules, hormones and antibodies between tissues and organs. Also, blood vessels work as a transport system for factors

that are produced by endothelial cells (ECs), particularly angiogenic factors, like vascular endothelial growth factor (VEGF).

The complex structure of the vascular network is achieved by the maturation of the immature blood vessels previously formed by angiogenesis or vasculogenesis (10). Vasculogenesis refers to the *in situ* formation of vessels orchestrated directly by endothelial precursor cells or angioblasts, whereas angiogenesis is defined as the process of new blood vessels formation through the sprouting of preexisting vasculature. Vasculogenesis is of major importance during embryonic development, but plays also a role in adulthood both in physiological, as well as in pathological conditions. On the other hand, angiogenesis plays a crucial role throughout postnatal life, being related to wound healing and the menstrual cycle (11, 12), inflammatory processes and also some pathological conditions, as rheumatoid arthritis, diabetic retinopathy, macular degeneration and tumor growth (13, 14), as well as accompanying growth repair, tissue remodeling and regeneration (6, 10, 15, 16).

The recruitment of new blood vessels through the activation of these two main processes of vascularization is still considered to be a great challenge in regenerative approaches. Different blood vessels exist, which express characteristic molecular markers. Endothelial precursors that form arteries express ephrin B2 (Efnb2), whereas its receptor, the B4 ephrin receptor (Ephb4), is preferentially expressed in veins (17). However, all blood vessels are formed by one thin inner layer of ECs, the endothelium, which is then covered by supporting cells, such as pericytes or smooth muscle cells and fibroblasts, which contribute for vessel stabilization and maturation. This vascular endothelium provides a selective barrier, separating the blood stream from the underlying tissues, with vascular wall cells being thus embedded in an extracellular matrix (ECM, Fig. 1.2).

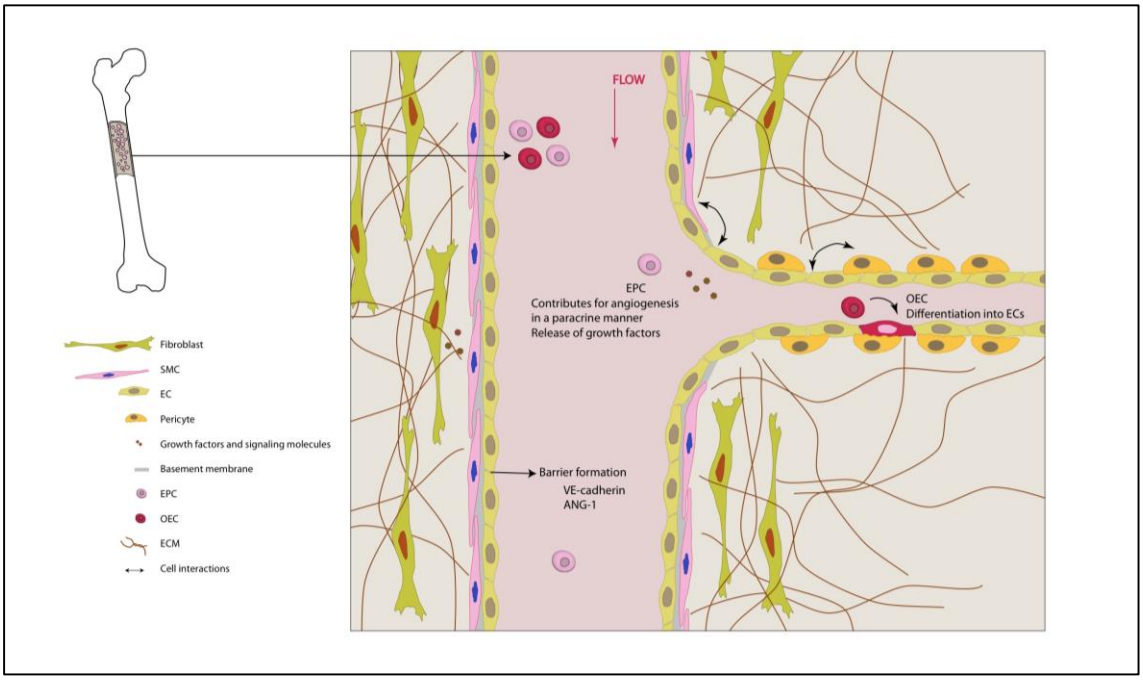


Figure 1.2. Schematic representation of the biological processes involved in vascularization, including cell-cell/ cell-matrix interactions. The vascular endothelium is formed by one layer of endothelial cells (EC). Their expression of adhesion molecules, like VE-cadherin and angiopoietin (Ang)-1, allows for intercellular interactions, which are important for vessel stabilization. Vascular maintenance is achieved through specific signaling (release of growth factors and signaling molecules) and direct physical contact between endothelial cells and mural cells, such as smooth muscle cells (SMC) and perycites, or fibroblasts. The extracellular matrix (ECM) will act as a bridge between blood vessels and the surrounding tissue. Moreover, different populations of endothelial progenitor cells exist in circulation – early endothelial progenitor cells (EPC) and outgrowth endothelial cells (OEC). These cells have distinct roles in vascularization: EPCs contribute for vascularization/angiogenesis in a paracrine manner through the release of growth factors, while OECs are able to contribute for the repair and formation of blood vessels by differentiating into mature ECs.

1.2.1. Endothelial cells

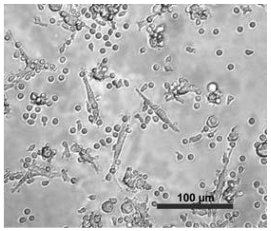
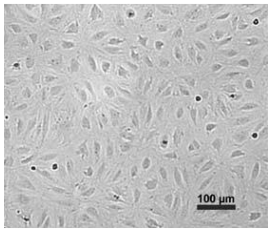
Endothelial cells (ECs) play a pivotal role in vascularization, forming the vascular endothelium. These cells characteristically express von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), and vascular endothelial (VE)-cadherin (18-20). However, molecular differences exist between endothelial cell populations due to the heterogeneity along this cell lineage, resulting in high variability when their functional behavior is assessed through angiogenesis assays (21). Some authors consider that mature endothelial cells (ECs) present a limited regenerative potential (22). The discovery of putative endothelial progenitor cells (EPCs) in 1997 (23) contributed to enlarge the knowledge of endothelial cell populations and to change the prevailing dogma until then, which stated that throughout postnatal life new blood vessels could only be generated by fully differentiated ECs. In fact, blood vessels are believed to be newly formed by endothelial precursors (15).

Although embryonic and adult stem cells exhibit a higher proliferative capacity (24), the use of EPCs has emerged as an alternative to them (25). EPCs exist in circulation

and have been described as being capable of incorporating into vessels after mobilization from bone marrow, in order to participate in neovascularization at sites of ischemia (23). These cells overcome some ethical considerations along with a deficient knowledge considering the control of embryonic stem cells differentiation (26). Two different types of EPCs are believed to exist, early EPCs and late EPCs, here called outgrowth endothelial cells (OECs) (20, 22, 27, 28), being commonly isolated from peripheral or umbilical cord blood. Other sources of endothelial progenitors include the adipose tissue (29, 30) and the amniotic fluid (31). Moreover, several works refer the bone marrow as a reservoir of EPCs (32-34), while others reported that these cells cannot be isolated from the bone marrow neither from cells mobilized from this organ (35). This finding defies the concept of bone marrow-derived circulating precursors of endothelial cells, originating further speculation about the eventual existence of a vascular source outside the bone marrow, or of a common precursor for endothelial progenitor cells and for hematopoietic stem cells. There is a great need to further explore these issues in future works, to improve knowledge on vascular biology, so that it can be applied to the development of tissue engineering strategies.

Despite their origin, these progenitor cells seem to be mobilized into circulation, contributing to the neovascularization process (24). However, there is still great controversy associated to the term “endothelial progenitor cell”. The main distinguishable features of both types of EPCs are briefly described in table 1.1. Early EPCs appear after 4 to 7 days in culture and have been described as bone marrow-derived cells, sharing surface markers expressed by hematopoietic stem cell populations, as CD14, CD45 and CD133 (20, 23, 36, 37), differentiating into phagocytic macrophages and possessing myeloid progenitor cell activity (38). On the other hand, OECs appear much later, after 14 to 21 days, exhibiting typical endothelial characteristics and being reported to incorporate into resident vasculature (22, 28, 39). Some authors reported that early EPCs failed to form interconnected vascular networks *de novo*, whereas OECs formed branching interconnected vascular networks after 72 hours in co-culture, being maximal after 14 days (22), which suggests a higher angiogenic potential associated to OECs (39). Moreover, early EPCs have been shown to preferentially express genes involved in immune responses and inflammation, while OECs expressed genes involved in development and angiogenesis, including the angiopoietin receptor Tie2, endothelial nitric oxide synthase (eNOS), ephrins and transforming growth factor (TGF)-β (20). A more recent study also showed that umbilical blood derived OECs secrete a broad spectrum of proinflammatory and angiogenic cytokines, including angiogenin, angiopoietin (Ang)-2 and platelet-derived growth factor (PDGF)-BB (40).

Table 1.1. Overview on characteristics that distinguish between EPCs and OECs. Adapted from Fuchs et al. 2010 (4) and Yoon et al. 2005 (41).

	EPCs	OECs	Marker functions
Morphology (4, 27, 41)	Spindle shaped morphology 	Cobblestone-like morphology 	
Appearance in culture	After 7 days in culture (4, 42)	After 2-3 weeks in culture (4, 27)	
Human phenotypic markers	CD31+ (22, 23)	CD31+ (18, 22)	Cell contact protein that mediates homotypic EC adhesion.
	CD45+ (22, 43)	CD45- (22, 43)	Surface transmembrane phosphatase expressed in hematopoietic lineage cells.
	CD34+ (23, 41)	CD34+ (41, 43)	Stem-cell-related marker.
	CD14+ (22, 41)	CD14- (22, 41)	Monocyte surface marker.
	CD146+ (22)	CD146+ (18, 22)	Adhesion molecule that mediates homotypic EC adhesion.
	CD133+ (44-46)	CD133- (43)	Stem-cell-related surface marker.
	Flt-1+ (42)	Flt-1+ (42)	VEGF receptor-1.
	eNOS (42)	eNOS (42)	Modulates VEGF-induced angiogenesis and vascular permeability *in vivo*.
	vWF+ (38, 42)	vWF+ (18, 38, 42)	Constitutive glycoprotein of the endothelium.
	VE-cadherin+ (42, 47)	VE-cadherin+ (18, 27, 42)	Cell contact protein that mediates homotypic EC adhesion.
	KDR+ (22, 42, 47)	KDR+ (22, 41, 42)	VEGF receptor-2.
	CD36+ (27)	CD36+ (27)	Receptor of TSP-1 and mediator of its anti-angiogenic activity
	Tie2+ (23, 48)	Tie2+ (48)	Cell surface receptor that bind and is activated by the angiopoietins.
	Caveolin-1- (20)	Caveolin-1+ (18, 20, 48)	Marker of endothelial differentiation. Upregulated during vessel formation.
	CD115+ (38)	CD115- (38)	Macrophage-specific antigen.
Proliferative potential	Low (22, 41, 42)	High (22, 41, 42)	
Tube formation	No (22, 42, 48)	Yes (22, 42, 48)	
Paracrine augmentation of angiogenesis	Yes (22)	No (22)	

1.2.2. Mural cells

Endothelial cells first associate to form tubules and afterwards mural cells are recruited allowing vessel maturation and stability (10). Signals involved in the recruitment and coverage of ECs by mural cells include PDGF-B, Ang1, TGF-β and NOTCH signaling (16).

Different mural cells exist depending on their morphology, location and the expression of specific markers, being divided into pericytes and smooth muscle cells (SMCs) (49) and contacting the microvascular basement membrane in different microvascular beds (50). Vascular SMCs are present in larger blood vessels, as arteries and veins, forming multiple concentric layers, whereas microvessels are covered by pericytes often organized in a discontinuous cell layer around the endothelial cell tube (49) and embedded within the vascular basement membrane (50) (Fig. 1.3).

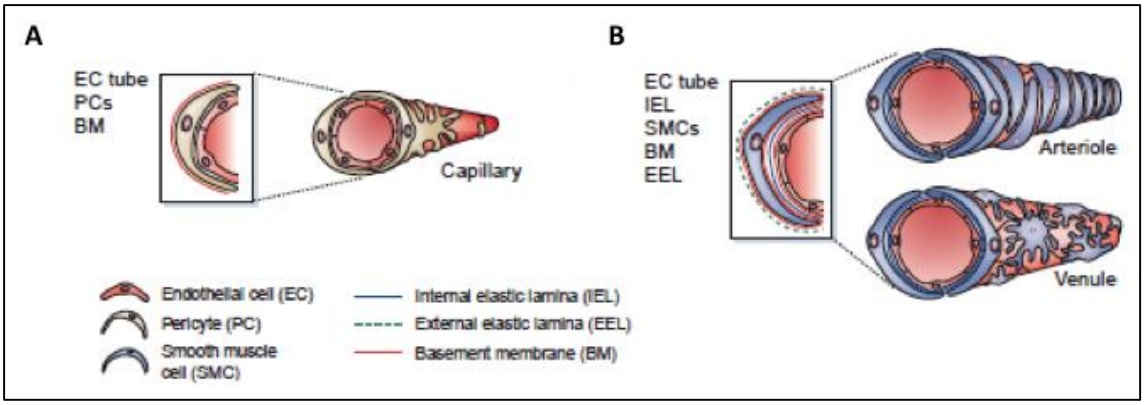


Figure 1.3. Schematic representation of blood vessels constitution. (A) Capillaries are the most abundant vessels in the human body, consisting of endothelial cells surrounded by basement membrane and a sparse layer of pericytes embeeded within the basement membrane. (B) Arterioles and venules are the vessels that will originate arteries and veins, respectively, and are covered by smooth muscle cells, having an increased coverage of mural cells, when compared to capillaries. Adapted from Jain (10).

These periendothelial cells contribute to stabilize blood vessels through direct physical contact (51), ECM deposition (52) and growth factors release, as VEGF and Ang-1 (16, 51, 52).

Different tissues exhibit varying mural cell morphology and different degrees by which they cover the endothelium (49).

1.2.3. Fibroblasts

Fibroblasts are mesenchymal cells and can be quite different in terms of cell dynamics, depending on their tissue of origin (53). They are the main source of ECM components (53, 54), including collagen I, fibronectin and proteoglycans (55). Human fibroblasts are abundant in the dermis and can be easily obtained from minimally invasive skin biopsies, using standard laboratory protocols (56, 57). Therefore, human dermal fibroblasts have been quite used for skin tissue engineering applications and have gained an increasing interest as a cell source for other applications, like neovascularization. Considering their location within the dermis, human dermal fibroblasts are divided into papillary (superficial dermis) and reticular (deep dermis) fibroblasts, exhibiting different characteristics in terms of cell morphology, production of ECM and growth factors, among others (58, 59). Although there is no specific marker to distinguish both fibroblasts, Janson et al. investigated the differences in gene expression patterns between these two types of fibroblasts, reporting that reticular fibroblasts showed an increased expression of genes involved in cell motility and contraction, including calponin 1 and transglutaminase 2, whereas papillary fibroblasts characteristically expressed genes involved in the immune response, such as netrin-1 and podoplanin (59). In what concerns to vascularization, a study reported that papillary fibroblasts were able to support the formation of highly branched tubular-like structures *in vitro*, while reticular fibroblasts were not (60). Therefore, human dermal fibroblasts might be used in cell therapies aiming for the revascularization of a damaged tissue.

For instance, the adventitial layer of large blood vessels is mainly composed by fibroblasts and associated ECM components (52). Besides, fibroblasts secrete potent angiogenic factors, like VEGF and fibroblast basic factor (FGF)-2, as well as matrix metalloproteinase (MMP)-2 and MMP-9 (61), which are proangiogenic metalloproteinases. Hence, it is possible that fibroblasts act as periendothelial progenitors *in vivo* (52), supporting EC survival and migration, as well as modulating the expansion of capillary-like networks, particularly *in vitro* (62). Moreover, since fibroblasts generate a scaffold for other cells through matrix deposition, they may alter the mechanical extracellular microenvironment, regulating vascularization processes (63).

1.3. The extracellular matrix

The ECM acts as a bridge between vascular wall cells and the respective surrounding tissues. Providing a three-dimensional (3D) support for EC proliferation and survival, ECM has a dual role – it acts as an adequate substrate for the organization of ECs into microvessels, simultaneously retaining and concentrating growth factors in the cellular microenvironment (61). ECM is therefore considered a reservoir of proteins that are involved in several physiological events, including wound healing and angiogenesis. The presence of proteases or protease inhibitors leads to changes in the bioavailability of matrix-sequestered factors (64).

Capillary morphogenesis is regulated through the immobilization of angiogenic cytokines, growth factors and other molecular cues involved in angiogenic activation (65), as well as by angiogenesis inhibitors (66). MMPs exist, which release pro-angiogenic (67) and anti-angiogenic peptides (68), through ECM cleavage (69). In particular, the endothelium is separated from the connective tissue by the basement membrane, a specialized layer of ECM where collagen IV and laminin, together with heparin sulfate proteoglycans, organize into networks, being both essential for basement membrane stability (70, 71). Collagen IV has been described as having the capacity to modulate the angiogenic response in a rat aorta model, resulting in the elongation of the neovessels (72). On the other hand, cleavage of collagen IV may result in the release of tumstatin and other angiogenesis inhibitors (66), showing the relevance of the ECM in regulating vascularization, among other physiological processes. In the case of laminins, they have been shown to be involved in the regulation of blood vessel diameter, rather than vascular development, in a model of embryoid bodies (71).

In addition, it is worth to mention that fibronectin, a component from the ECM of developing microvessels, also acts as a scaffold for cell adhesion and migration (73, 74) and plays a role in the elongation of these developing microvessels (75).

Moreover, ECM stiffness has also been proved to be essential for lumen formation by ECs (76).

Given that ECM proteins also possess binding sites for cells to adhere through their surface receptors, ECM acts as a key controller of cell behavior, activating several intracellular signaling pathways and, in turn, cells degrade and remodel the ECM (65, 70, 77). Hence, understanding cell-matrix interactions is of major importance to mimic the natural ECM when developing functionalized biomaterials that provide biospecific cell adhesion and control cellular functions (78), since the ECM corresponds to a natural scaffold for tissue development and repair, supporting tissue reconstruction (79).

1.4. Cellular crosstalk and communication: the case of co-culture systems

Co-culture systems may have an important role in what refers to cellular crosstalk, namely through the production of growth factors and ECM synthesis. The importance of a co-culture system relies on the intricate communication pathways that are established between different cell types, both through diffusible signaling molecules and by cell-cell contacts. In fact, angiogenesis is controlled by the interaction between ECs and other cell types (80) and different works have shown that cells expressing smooth muscle cell/ pericyte markers, such as α-smooth muscle actin (α-SMA), appeared in close proximity to capillary-like structures (26, 52, 81, 82). Hence, this section will focus on the use of ECs in co-culture systems to promote vascularization in tissue engineering.

1.4.1. Co-cultures of ECs with pericytes

Pericytes have been recently described as $CD146^+$ $CD34^-$ cells; however, these markers are not reliable *in vitro* and functional assays are needed (83). These cells were considered a multipotent subpopulation of mesenchymal stem cells, being able to retain endothelial tubules over time and to stabilize endothelial networks on Matrigel (83).

Pericytes have been investigated in co-culture systems with endothelial cells to mimic the blood brain barrier (84, 85), or the gliovascular complex from the neurovascular unit (86), as well as the retinal microvascular environment to study diabetic retinopathy (87). Although pericytes are described as being capable of protecting the blood brain barrier from disruption, for instance, following short periods of hypoxia (84), works in the field of vascularization are still scarce. Some authors developed *in vitro* models of the gliovascular complex and reported that capillary-like structures are maintained over time due to the presence of pericytes in co-cultures of endothelial cells and astrocytes (86), but pericytes are not the focus of these research models.

On the other hand, the 10T1/2 cell system, a mouse embryonic cell line, has been used as a precursor of pericytes or SMCs, since their differentiation can be induced by TGF-β (88). By co-culturing these cells with HUVECs, TGF-β produced by HUVECs induced the differentiation of 10T1/2 into pericytes and pericytes were shown to produce VEGF, stimulating ECs to organize into capillary-like structures, with markers of activated pericytes, like aminopeptidase N, being detected after the first 24h of culture (88).

The same pericyte precursors were also used by Au et al. to investigate whether the implantation of higher cell densities of cord blood(CB)-derived EPCs could obviate the need for co-implantation (89). Although some of the implanted CB-EPCs aligned into blood vessels, these vessels were only transiently perfused and regressed after 23 days,

with 10T1/2 cells functioning as perivascular cells *in vivo* (89). Therefore, co-implantation of endothelial and perivascular cells has been demonstrated to be critical for *in vivo* capillary formation, maintenance and stability. Despite the fact that vascularization starts with capillaries and progressively increases complexity in terms of cell organization, co-culture models have been focusing on cells that are mainly present in large vessels, instead of aiming to understand the role of pericytes in supporting vascularization. Therefore, further studies are needed, in order to standardize protocols concerning the use of a true pericyte and to use these cells for tissue engineering.

1.4.2. Co-cultures of ECs with smooth muscle cells

SMCs are known to be involved in the stabilization and maturation of blood vessels, being crucial for the development of functional blood vessels. A recent study has shown that implantation of decellularized small intestinal mucosa scaffold reseeded with HUVECs and SMCs resulted in a rapid vascularization (4 days) of the graft, together with the maintenance of favorable mechanical properties of the tissue engineered vascular grafts (90).

Co-administration of ECs and SMCs has been investigated in models of ischemia. For instance, Foubert et al. implanted endothelial and smooth muscle progenitor cells (OECs and SMPCs, respectively) in combination into an ischemic leg of nude mice and verified that this combined administration resulted in a higher microvascular density, compared to OECs implanted alone (91). In addition, molecular events that are involved in vascularization steps appear also seem to be different in co-culture systems, compared to ECs cultured alone. For instance, PDGF-B, a factor involved in mural cell recruitment, was found to be downregulated over time in co-culture spheroids of HUVECs/SMCs, contrasting to HUVECs alone, which suggests that co-cultures properly mimic the *in vivo* phenotype (92), since expression of PDGF-B is believed to be restricted to immature capillaries. Also, the Ang1/Tie-2 system regulates both vascular quiescence and angiogenesis. Foubert et al. have demonstrated the involvement of these signaling partners in the formation of capillary-like networks in co-cultures. In fact, SMPCs released Ang-1 and its receptor Tie-2 was subsequently activated in OECs, showing that paracrine release of Ang-1 modulate OEC incorporation into the vascular endothelium (91).

Given that OECs are thought to exhibit better characteristics in what concerns to vascularization in tissue engineering, Melero-Martin and colleagues first demonstrated the *in vivo* vasculogenic potential of OECs by performing a co-implantation protocol of

OECs from the cord blood or from the adult peripheral blood together with SMCs in Matrigel plugs by subcutaneous injection into immunodeficient mice (26). This approach resulted in the formation of human EC-lined vessels, which contained murine erythrocytes, with functional anastomoses being observed (26). They have shown that blood vessel formation occurred only for co-implantation experiments (26), reinforcing the importance of cellular crosstalk.

1.4.3. Co-cultures of ECs with fibroblasts

As previously mentioned, the role of fibroblasts in tissue regeneration has somehow been underestimated in tissues other than skin (93). However, in the past decade, different works have focused on the potential of fibroblasts to enhance vascularization and EC assembly into tubular structures. The implantation of neonatal human dermal fibroblasts using Matrigel plugs in mice showed that fibroblasts induced the ingrowth of blood vessels from the host vasculature (93), thus suggesting that these cells take part in the recruitment of ECs *in vivo*. A combination of fibroblast-derived proteins, comprising Ang-1, angiogenin, hepatocyte growth factor, TGF-α and tumor necrosis factor (TNF), has been described to support EC sprouting, while matrix proteins, such as collagen I, secreted protein acidic and rich in cysteine (SPARC) and insulin-like growth factor-binding protein 7 (IGFBP7), among others, were necessary for lumen formation in 3D fibrin gels, which proved to be related to an increased matrix stiffness (76). In particular, when ECM synthesis by fibroblasts is reduced, EC tube formation decrease, although ECM synthesis seems not sufficient for the organization of capillary-like structures, which requires a close association between ECs and living fibroblasts (61). It has also been described that pericytes spontaneously originate from fibroblasts, when co-culture systems with ECs are established (81). Berthod et al used a human tissue engineered skin model to perform co-cultures of ECs and fibroblasts, showing that cells expressing α-SMA only appeared around capillary-like structures, which reinforces the role of fibroblast-derived pericytes as supporting cells in vessel formation (81). In addition, a spheroid co-culture model has demonstrated that ECs were capable of attaching and migrating along fibroblasts-derived ECM into spheroids in order to form a capillary-like network (62). Other work reported a rapid sprouting of HUVECs seeded on microcarrier beads and co-cultured with dermal fibroblasts, in which capillary-like networks formed in a fibrin-based tissue construct after 2-3 days and continued to remodel up to 14 days (2). On the other hand, when neonatal human dermal fibroblasts were immobilized in alginate gels grafted with RGD peptidic sequence and co-cultured with HUVECs, it was

observed that fibroblasts had the ability to modulate and support the assembly of ECs into capillary-like structures (94).

In addition, the simultaneous use of bioactive silicate materials together with co-cultures of fibroblasts and HUVECs resulted in an enhanced formation of highly anastomosed capillary-like structures, with calcium silicate extracts inducing VEGF expression by fibroblasts (95).

Another relevant aspect is the contribution of OECs to accelerate wound healing for skin applications. In fact, OECs integrated into dermal fibroblast layers were capable of actively incorporating into new blood vessels, promoting re-oxygenation of the wound bed (96).

The implantation of fibroblasts in co-culture with OECs using 3D tissue constructs has also been described. Chen and colleagues created prevascularized tissue constructs by mixing endothelial cells and fibroblasts in a fibrinogen solution polymerized through the addition of thrombin (97). After OECs being organized into capillary-like networks *in vitro*, fibrin-based tissue constructs were implanted, with blood perfusion and the formation of anastomosis between vessels from the implanted structure and the host vasculature being observed within 27h after implantation, when OECs were co-cultured with a high density of fibroblasts (98).

Considering these data altogether, further studies should be performed using co-cultures of OECs and fibroblasts, in order to understand the molecular mechanisms underlying this cellular crosstalk during the angio- /vasculogenic processes.

1.6 Hypothesis and objectives

Since activation and migration of fibroblasts is required for several physiological events that rely on angiogenesis, the hypothesis underlying the research reported in the present work is that outgrowth endothelial cells and mature endothelial cells respond differently when co-cultured with different types of fibroblasts, in what concerns the formation of capillary-like structures.

In vitro work was performed in order to accomplish the following objectives:

- To isolate and characterize populations of outgrowth endothelial cells and to evaluate their angiogenic potential;
- To investigate the ability of two types of human dermal fibroblasts to induce/ support the formation of vascular-like networks by macrovascular endothelial cells and outgrowth endothelial cells;

- To characterize the extracellular matrix that is being produced over time in these co-culture systems.

CHAPTER II

MATERIALS AND METHODS

2.1. Cell culture

2.1.1. Isolation and expansion of human outgrowth endothelial cells

Umbilical cord blood (UCB) samples were collected from healthy donors from Hospital de São João (Porto, Portugal). All samples were obtained under informed consent, according to the Declaration of Helsinki and to the ethical committee of Hospital de São João. UCB (volume of 80-100 mL/ isolation) was collected using blood bags (Mollitia, Fig. 2.1A) and processed as soon as possible after baby delivery (within an hour, at maximum).

Human OECs were isolated from cord blood samples, according to protocols already established (99). UCB was first diluted 1:1 (v/v) in Hank's Balanced Salt Solution (HBSS, Sigma) and overlaid onto Histopaque-1077 solution (Sigma) for separation of mononuclear cells (MNCs). After centrifugation, the MNCs fraction was collected from the buffy coat layer (Fig. 2.1B) and washed with HBSS. After another centrifugation step at 300g for 10 min, the supernatant was discarded and cells were resuspended in Microvascular Endothelial Cell Growth Medium-2 (EGM-2MV, Lonza) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS, Sigma) and centrifuged again for 7 min. Finally, MNCs were platted in type I collagen-coated 6-well tissue culture plates (BD, Biosciences) in a cell density of $10x10^6$ cells/well. After 24h, wells were washed with phosphate buffered saline (PBS) and nonadherent cells were discarded, while adherent cells were cultured again in EGM-2MV supplemented with 10% FBS. The medium was

changed every other day until colonies with a cobblestone-like morphology appeared, which took up to 2 – 3 weeks. These cells, the so-called OECs, were collected and expanded over several passages and characterized through imaging flow cytometry, immunocytochemistry and western blot. For each experiment, OECs were used at passages 2-4.

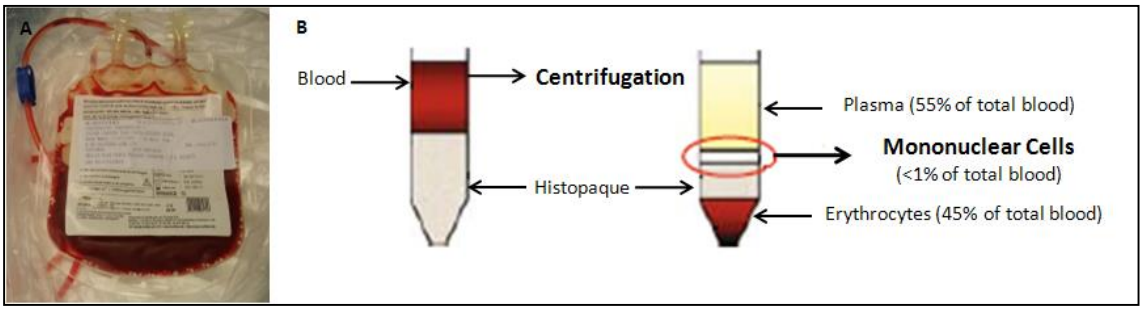


Figure 2.1. Isolation of OECs from umbilical cord blood. (A) Blood bag used to collect umbilical cord blood after baby delivery. (B) Schematic representation of the separation of blood elements using Histopaque-1077. Erythrocytes and granulocytes form a red pellet at the bottom of the centrifuge tube; MNCs form a fine band at the interface between Histopaque-1077 and plasma, which appears at the top of the tube.

2.1.2. Culture of HUVECs

Human umbilical vein ECs (HUVECs) were kindly provided by Professor James Kirkpatrick from REPAIR-lab (University of Mainz, Germany) after being isolated as previously described (100). These cells were cultured in Endothelial Cell Growth Medium-2 (EGM-2, Lonza) supplemented with 5% (v/v) inactivated FBS (Sigma) and maintained in a humidified atmosphere with 5% CO_2 – 95% air at 37ºC and medium was changed twice a week until 90% confluence was reached, when cells were trypsinized and a split ratio of 1:3 was performed. For each experiment, HUVECs were used at passages 2-4.

2.1.3. Culture of fibroblasts

Neonatal human dermal foreskin fibroblasts-1 (HFF-1) were obtained from American Type Culture Collections (ATCC) and cultured according to supplier's instructions. Juvenile human dermal fibroblasts (HDF) were kindly provided by Professor James Kirkpatrick from REPAIR-lab (University of Mainz, Germany).

Both types of fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 15% (v/v) inactivated FBS (Sigma) and 1% (v/v) antibiotic/antimycotic solution (AB/AM, Sigma). Cells were maintained at 37ºC in a

humidified 5% CO_2 atmosphere and medium was changed twice a week until 90% confluence was reached, when cells were trypsinized and a split ratio of 1:3 - 1:5 was performed (94). For each experiment, fibroblasts were used at passages 8-10.

2.2. Characterization of OECs by imaging flow cytometry

To characterize OECs through imaging flow cytometry, cells were seeded in 0.2% (m/v) gelatin-coated 25 cm^2 flasks and cultured in EGM-2MV until 90% confluence was reached, when cells were trypsinized and counted. Cells were manipulated in suspension and, therefore, centrifugation steps (1200 rpm for 5 min) were performed between every incubation and washing steps. Cells were washed in PBS, fixed with 4% (v/v) of paraformaldehyde (Sigma) and permeabilized with 0.2% (v/v) Triton-X 100 (Merck) in PBS. After, cells were stained against CD31 (mouse anti-human CD31, Dako, 1:100), CD34 (mouse anti-human CD34, Dako, 1:50), CD144 (mouse anti-human CD144, BD Pharmingen, 1:100) and Flk-1 (mouse anti-human Flk-1, Santa Cruz Biotechnology, 1: 200). Alexafluor 488 goat anti-mouse (BD Pharmingen, 1:1000) was used as the secondary antibody. Samples were analyzed on imaging flow cytometer ImageStream® (Amnis), acquiring at least 10000 events. The data was analyzed using the IDEAS® software (Amnis).

2.3. Assembly of OECs into capillary-like structures by Matrigel assay

In order to evaluate the capacity of OECs to organize into capillary-like structures, a Matrigel assay was performed. For this, 200 µL of Growth Factor Reduced Basement Membrane Matrix (GFR-Matrigel, BD Biosciences) were added to a 24-well culture plate and incubated at 37ºC for 30 min, to allow for Matrigel to solidify. Then, $2x10^4$ of OECs in 500 µL of EGM-2MV were added per well. After, wells were maintained at 37ºC in a humidified 5% CO_2 atmosphere. The cells were monitored using an inverted light microscope, to observe the appearance of capillary-like structures, which were then counted.

2.4. Direct contact co-cultures of endothelial cells and fibroblasts

To evaluate the influence of fibroblasts in the ability of endothelial cells to form capillary-like structures, single-cell suspensions were seeded as mixtures. Co-cultures of ECs (HUVECs or OECs) with fibroblasts (HFF-1 or HDF) were established using a

cell ratio of 2:1. Cells were seeded onto 0.2% (m/v) gelatin-coated glass coverslips on 24-well ($2x10^4$ ECs: $1x10^4$ Fibroblasts) and 6-well ($2x10^5$ ECs: $1x10^5$ fibroblasts) plates coated with 0.2% (m/v) gelatin (Merck) for immunofluorescent staining and protein extraction, respectively. ECs and fibroblasts were seeded at the same time to the culture plate and were grown in EGM-2 culture media. Different time points were considered – 7, 14 and 21 days. After each time point, cells were fixed and immunostained as described below. Co-cultures were then observed under a confocal microscope (CLSM, Leica SP2 AOBS; Leica Microsystems), photographed and parameters as the number of capillary-like structures, length, thickness and the number of branching points were determined.

2.5. Characterization of cell phenotype and ECM by immunocytochemistry

For characterizing cells by immunofluorescence, the following primary antibodies were used: mouse anti-human CD31 (Dako, 1:100), rabbit anti-human vWF (Dako, 1:8000), mouse anti-human CD34 (Dako, 1:50), mouse anti-human CD144 (VE-cadherin, BD Pharmingen, 1:100), mouse anti-human Flk-1 (VEGFR2, Santa Cruz Biotechnology, 1: 200), mouse anti-human α-SMA (Dako, 1:100), mouse anti-human podoplanin (Santa Cruz Biotechnology, 1:100). For evaluating the distribution and organization of the extracellular matrix, mouse anti-human collagen IV (Dako, 1:100), mouse anti-human fibronectin (Antibody Shop, 1:200), rabbit anti-human laminin (Sigma, 1:1000) and mouse anti-human collagen I (abcam, 1:2000) were used. Alexafluor 488 goat anti-mouse (BD Pharmingen, 1:1000) and Alexafluor 555 donkey anti-rabbit (BD Pharmingen, 1:1000) were used as secondary antibodies. All antibodies were diluted in 1% (m/v) bovine serum albumin (BSA, nzytech) /PBS.

After being cultured for specific times, cells were fixed with 4% (v/v) of paraformaldehyde (Sigma) for 30 min at room temperature. 0.2% (v/v) Triton-X 100 (Merck) in PBS was used for cell permeabilization. Cells were washed in PBS and incubated for 1h at room temperature with primary antibodies. After washing three times with PBS, cells were incubated for 1h at room temperature with the secondary antibodies. Cell nuclei were then counterstained with 1µg/mL DAPI (Roche) in PBS for 15 min. Finally samples were washed with PBS and examined by fluorescence microcopy (Axio Zeiss Observer Z.1, USA).

2.6. Protein extraction

For protein extraction, cells were trypsinised, centrifuged and suitable amounts of RIPA buffer (RIPA buffer 10x, Millipore, diluted 1:10 in Millipore water; phosphatase inhibitor cocktail, *Sigma* and complete protease inhibitor cocktail, *Roche*) were added to the cell pellets on ice. After recovering the supernatant, a BCA (bicinchoninic acid) protein Assay Reagent Kit (Pierce, Thermo Scientific) was used to determine the protein concentration according to the manufacturer's protocol. Proteins were quantified using a microplate reader (Thermo, Electron Corporation) at 550 nm.

2.7. Western blot analysis for cell characterization and ECM components quantification

For cell characterization, the following primary antibodies were used: rabbit anti-human β-actin (abcam, 1:3000), rabbit anti-human CD31 (abcam, 1:1000), mouse anti-human α-SMA (Dako, 1:1000), mouse anti-human podoplanin (Santa Cruz Biotechnology, 1:1000), rabbit anti-human transglutaminase-2 (Genetex, 1:1000), rabbit anti-human Flk-1 (VEGFR2, Cell Signaling, 1:1000) were used. For quantifying ECM components, mouse anti-human collagen IV (Dako, 1:500) and rabbit anti-human laminin (Sigma, 1:1000) were used. β-actin was used as an internal control. Goat anti-rabbit IgG HRP (Santa Cruz Biotechnology, 1:2000), goat anti-mouse IgG HRP (Santa Cruz Biotechnology, 1:2000), donkey anti-rabbit IgG-B (Santa Cruz Biotechnology, 1:2000) and goat anti-mouse IgG-B (abcam, 1:2000) were used as secondary antibodies.

Proteins were separated according to their molecular weight using SDS polyacrylamide gel electrophoresis (SDS-PAGE). For that, approximately 10 µg of cell proteins were mixed with loading buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% Glycerol, 0.04% Bromophenol blue (1:4) and dithiothreitol (DTT 1M, 1:20)), being then denatured at 99ºC for 1 min. After, samples were loaded into the wells of the stacking gel and protein separation was performed at 200 V in SDS-running buffer (125 mM Tris HCl pH 8.3, 96 mM Glycine, 0.5% SDS). Precision Plus Protein™ Dual Color Standards (Bio-Rad) were used as protein standards. In order to make the proteins accessible for antibody detection, separated proteins were transferred from the gel onto a nitrocellulose membrane (Amersham Biosciences), using a mini transfer chamber filled with SDS transfer buffer (25 mM Trizma, 192 mM glycine, 20% methanol) for 1h at 40 V. Subsequently, the membrane was blocked in blocking solution for 1h at room temperature, washed 6 times with TBS 0.05% Tween (TBST) for 5 min and incubated with the primary antibody overnight at 4ºC. After another washing step of 6 times with

TBST for 5 min, each membrane was incubated with the secondary antibody for 1h at room temperature. All antibodies were diluted in the blocking solution.

For detecting proteins with lower expression, membranes were incubated with avidin-biotin complex solution (VECTASTAIN ABC kit PK-6100, Vector Laboratories) for 30 min.

Antibodies were detected using enhanced chemiluminescent (ECL) reagents (GE Healthcare) and the membranes were visualized using ChemiDoc™ MP System (Bio-Rad). Images were acquired and western blots were quantified using Image Lab Software 4.0.1 (Bio-Rad). Each sample was assayed three times in separate gels. Results are presented as relative protein expression normalized to signal intensity of β-actin protein.

2.8. Preparation of fibroblast conditioned media

Fibroblasts (either HFF-1 or HDF) were cultured in DMEM (Sigma) supplemented with 15% (v/v) FBS (Sigma) and 1% (v/v) AB/AM solution (Sigma) until 70% confluence was reached. Then, media was removed and cells were washed in PBS. Conditioned culture media was obtained by culturing fibroblasts during 24h in EBM-2 (Basal Medium) supplemented with 5% (v/v) FBS (Sigma) and 1% (v/v) AB/AM solution (Sigma). Medium was collected, centrifuged at 1200 rpm for 5 min. and the supernatant was harvested at -20ºC.

2.9. Determination of metabolic activity by MTS assay

Endothelial cells (HUVECs and OECs) were separately plated at a density of $6x10^4$ mL^{-1}, left to adhere for 24h and, then, cultured in fibroblast conditioned media (CM) using a final volume of 100 µL. Different percentages of CM were used – 25, 50, 75 and 100%. Fresh culture medium was used to complete the volume. After 24h, a colorimetric method was used to evaluate EC metabolic activity (CellTiter 96^R AQ_{ueous} One Solution Cell proliferation Assay, Promega), according to supplier's instructions. The absorbance was measured at 490 nm in a plate reader (Thermo, Electron Corporation). The quantity of formazan product as measured by this absorbance is directly proportional to the number of living cells in culture.

2.10. Co-cultures of endothelial cells and fibroblasts in Matrigel

HDF were cultured for 24h at a cell density of 10000 cells/well in a 24-well plate. Matrigel assay was performed similarly to the assay previously described. Here, 200

µL/well of GFR-Matrigel (BD Biosciences) were added to the top of the fibroblast monolayer. Then, 60000 endothelial cells were added per well and cultured in 500 µL of EGM-2 culture medium. Controls were established by omitting fibroblasts from the experimental setup (Fig. 2.2). During the experiment, cells were monitored and photographed using an inverted light microscope, to observe the appearance of capillary-like structures. The number of capillary-like structures, length, thickness and the number of branching points were also evaluated.

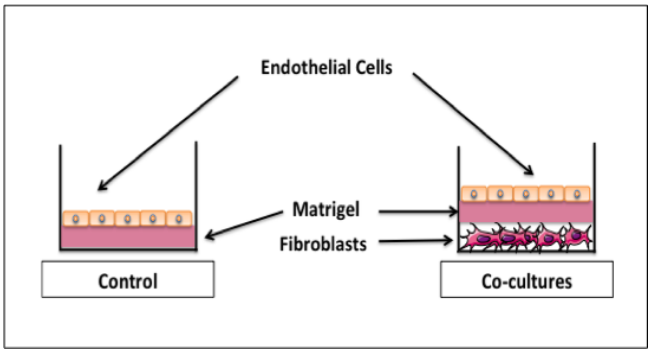


Figure 2.2. Experimental design for co-culturing endothelial cells and fibroblasts in Matrigel.

2.11. Imaging and image quantification

Cell morphology and adhesion were monitored daily using an optical microscope. Cell characterization by immunofluorescence was analyzed using a Carl Zeiss Axiovert inverted microscope. Monocultures and co-cultures were visualized and scanned under a Leica confocal microscope (CLSM, Leica SP2 AOBS; Leica Microsystems) using laser wavelengths of 405 nm (DAPI), 488 nm (green) and 561 nm (red). Image analysis software ImageJ64 was used for quantifications, including the length and diameter of capillary-like structures.

2.12. Statistical analysis

All experiments were performed in triplicate. Quantifications are expressed as mean ± standard deviation (SD). The Student's t-test was used for comparisons between two groups. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $p<0.05$.

CHAPTER III

RESULTS

3.1 Endothelial phenotype of OECs from umbilical cord blood

Outgrowth endothelial cells (OECs) were isolated from human umbilical cord blood and appeared after 2-3 weeks as small colonies in monocultures of MNCs, developing a characteristic cobblestone-like morphology over time (Fig. 3.1), similarly to endothelial cells, as well as showing a high proliferative potential. These cells were characterized in terms of the expression of endothelial markers, including CD31, VE-cadherin and vWF, as well as VEGFR2 and CD34 (Fig. 3.2A). CD31 and VEGFR2 expression was also verified at the protein level (Fig. 3.2B). These cells expressed significant levels of CD31 and VEGFR2, identical to β-actin, housekeeping gene. The presence of CD31 and VEGFR2 markers confirms that OECs are an endothelial cell population, while CD34 confirms that these cells have a mesodermal origin and that they are still in an endothelial progenitor cell state.

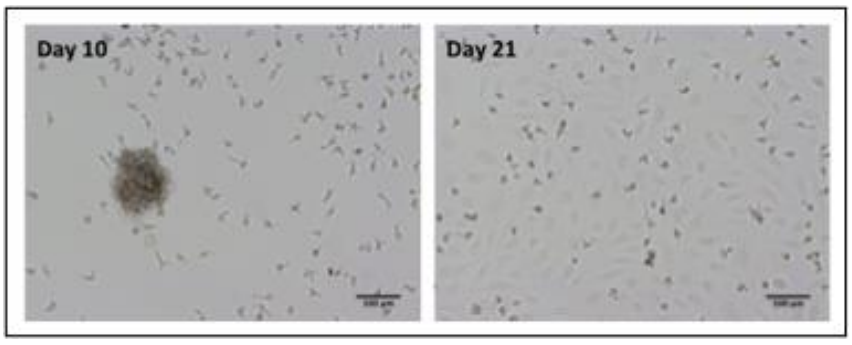


Figure 3.1. Morphology of outgrowth endothelial cells in culture. Here, at day 10, OECs are differentiating as colonies and at day 21 cells are confluent in the well, exhibiting a cobblestone-like phenotype. Scale bar, 100 μm.

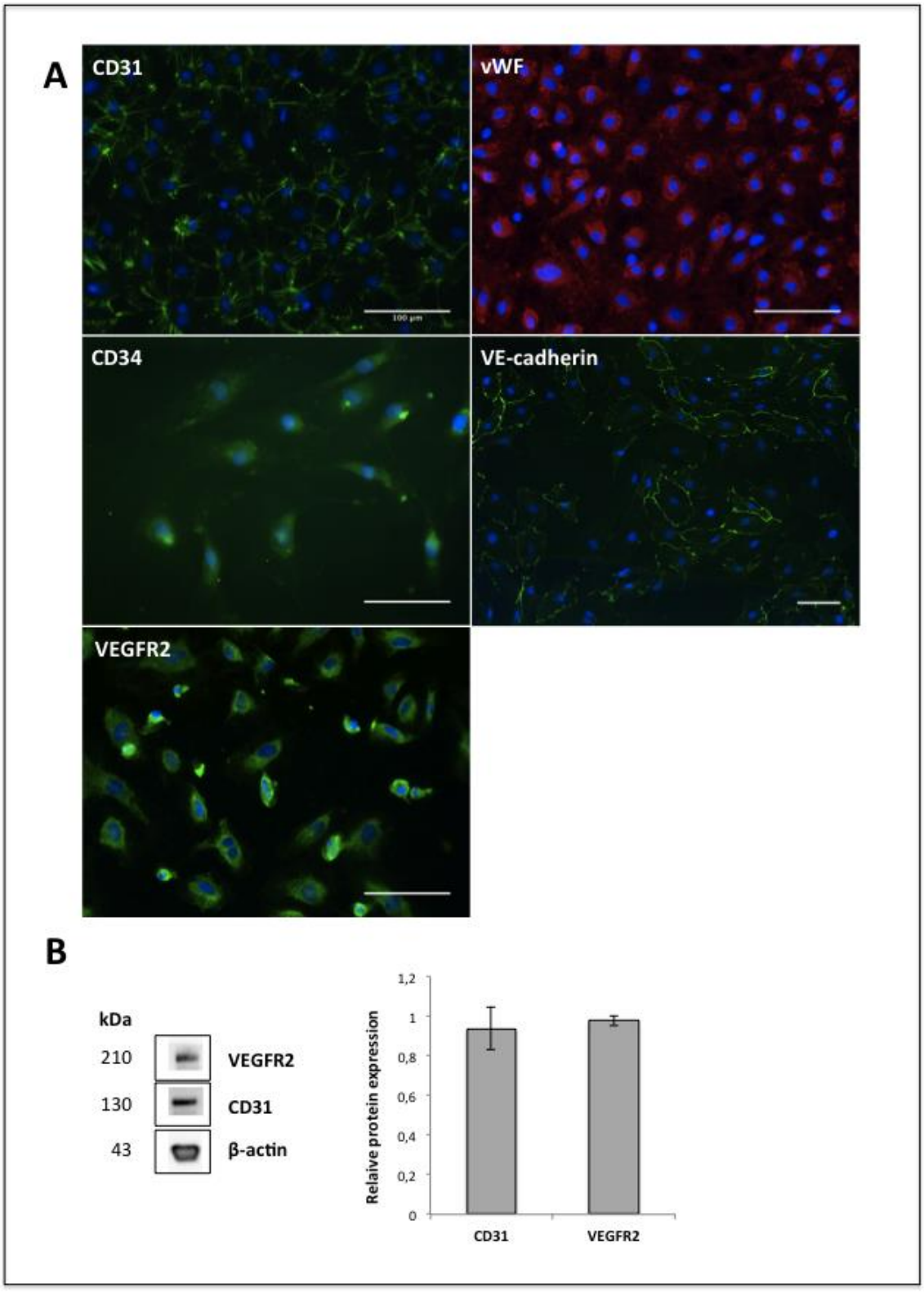


Figure 3.2. Immunophenotypic characterization of OECs. (A) Characterization of OECs by immunocytochemistry. OECs stained positive for CD31 (green), vWF (red), CD34 (green), VEGFR2 (green) and VE-cadherin (green). Nuclei are counterstained with DAPI (blue). Scale bars, 100 µm. (B) Expression of CD31 and VEGFR2 in OECs determined by western blot analysis. Relative protein expression was obtained through normalization to signal intensity of β-actin protein (n=3).

Table 3.1 summarizes the percentage of cells expressing CD31, CD34, VE-cadherin and VEGFR2, with over 99% of the cells used in this study staining positive for CD31 and VEGFR2, while approximately 91% of this cell population was positive for CD144 (VE-cadherin). The overall expression of CD34 was markedly lower than the expression of the other cell markers, with only approximately 33% of this cell population staining positive for CD34.

Table 3.1. Phenotypic characterization of OECs by imaging flow cytometry. OECs were immunostained against CD31, CD34, CD144 (VE-cadherin) and VEGFR2. The percentage of positive cells was calculated according to the gate defined for the unstained controls.

	OECs			
	CD31	**CD34**	**CD144**	**VEGFR2**
Positive (%)	99.79	33.48	91.39	99.3

In addition, by establishing a relation between the median pixel (a measure of signal strength) and a texture feature, the gradient RMS, imaging flow cytometry allowed to characterize this cell population in terms of the cellular distribution of the different studied markers.

Indeed, OECs used in the present study appeared to constitute an heterogeneous population, making possible to define two to six distinct regions, according to the cellular distribution and intensity of each marker (R1-R6, Fig. 3.3). The population of $CD31^+$ cells (Fig. 3.3A), as well as $CD34^+$ cells (Fig. 3.3B), could be divided into six regions (R1-R6), with the expression of both surface markers varying from an homogeneous distribution with low intensity (R1) to an heterogeneous distribution with high intensity (R6). On the other hand, the expression of VE-cadherin (CD144, Fig. 3.3C) and VEGFR2 (Fig. 3.3D) could only be divided into a region of homogeneous distribution with low intensity (R1) and a region of heterogeneous distribution with low intensity (R2), although a single $CD144^+$ cell appeared with a higher intensity (R6, Fig. 3.3C).

Considering these characterization data together, it is possible to define OECs used here as an heterogeneous cell population, expressing relevant EC markers, like CD31, vWF, VE-cadherin, VEGFR2 and, to a lower extent, CD34.

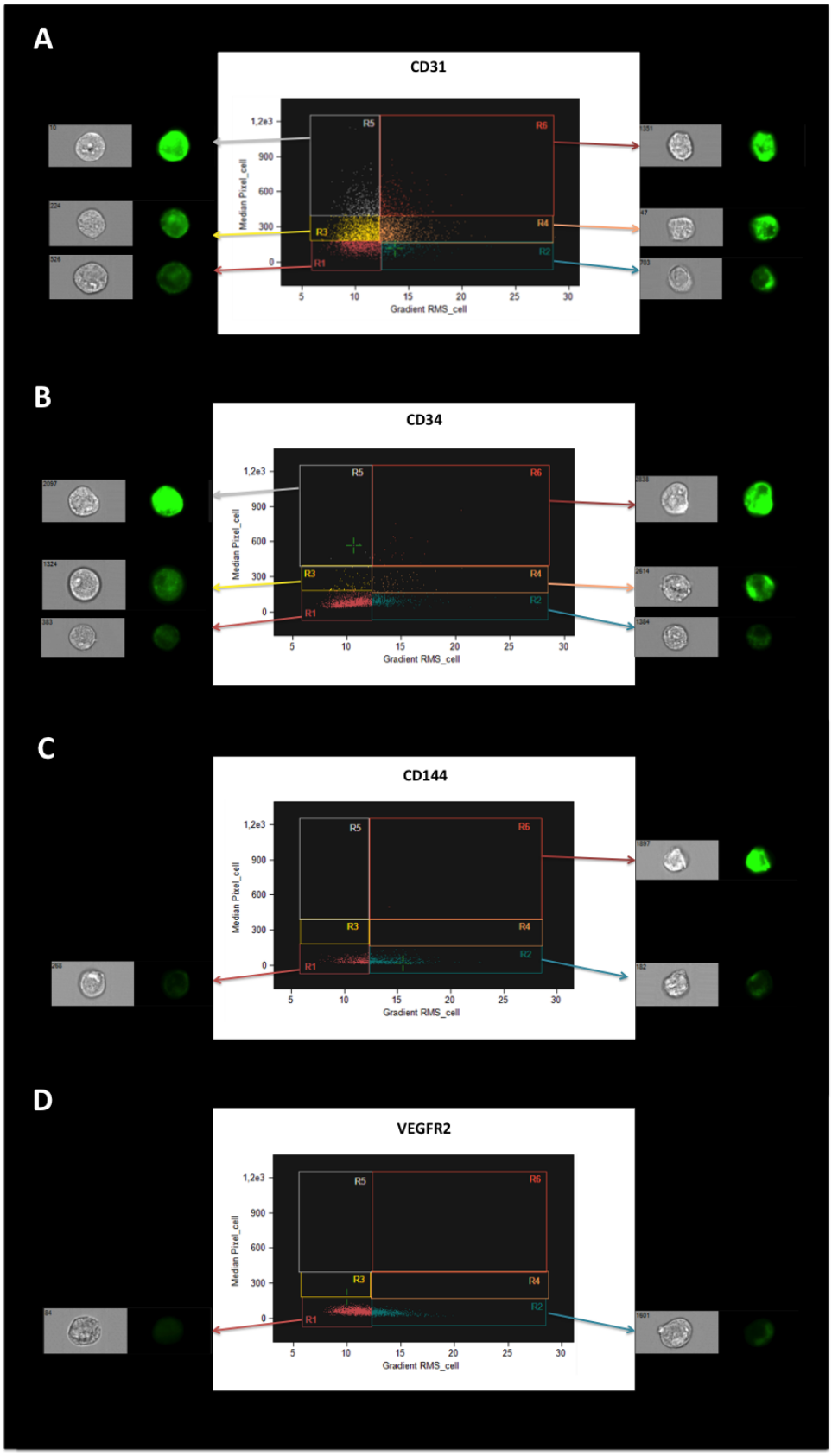


Figure 3.3. Characterization of the phenotypic heterogeneity of OECs by imaging flow cytometry in terms of the positive expression of CD31, CD34, CD144 and VEGFR2. Results presented here correspond to a relation between the median pixel (signal strength feature) and the gradient RMS (texture feature).

In addition, GFR-Matrigel™ assay was performed in order to evaluate the ability of OECs to assemble into capillary-like structures, as this is another endothelial characteristic. Indeed, OECs were capable of organizing into typical polygonal structures (Fig. 3.4A). This was observed as soon as 6h after OECs being seeded on top of a GFR-Matrigel™ layer and these capillary-like structures were maintained at least until 48h of culture, with a decrease in their number being observed over time (Fig. 3.4B).

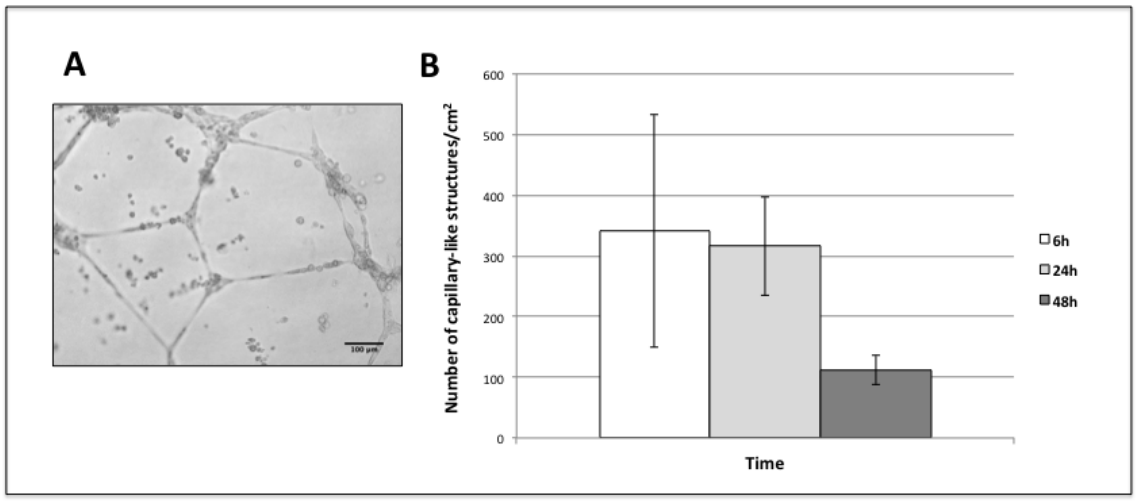


Figure 3.4. Assembly of OECs into capillary-like structures in Matrigel. (A) Microscopic image of capillary-like structures formed by OECs after 24h in Matrigel. Scale bar, 100 μm. (B) Number of capillary-like structures/cm^2 formed by OECs in Matrigel after 6, 24 and 48h.

3.2 Effect of different culture media in EC metabolic activity

In order to determine the influence of different culture media in the metabolic activity of endothelial cells, an MTS assay was performed. For that, EGM-2 and EGM-2MV were prepared according to manufacturer's instructions and supplemented with 5% and 10% FBS, respectively. After 24h in culture, no differences were observed between the behavior of both types of ECs cultured either in EGM-2 or EGM-2MV (Fig. 3.5). In order to facilitate the comparison with other works, EGM-2 culture medium was selected to perform the subsequent angiogenesis assays, as it is widely used for establishing different co-culture systems aiming for vascularization.

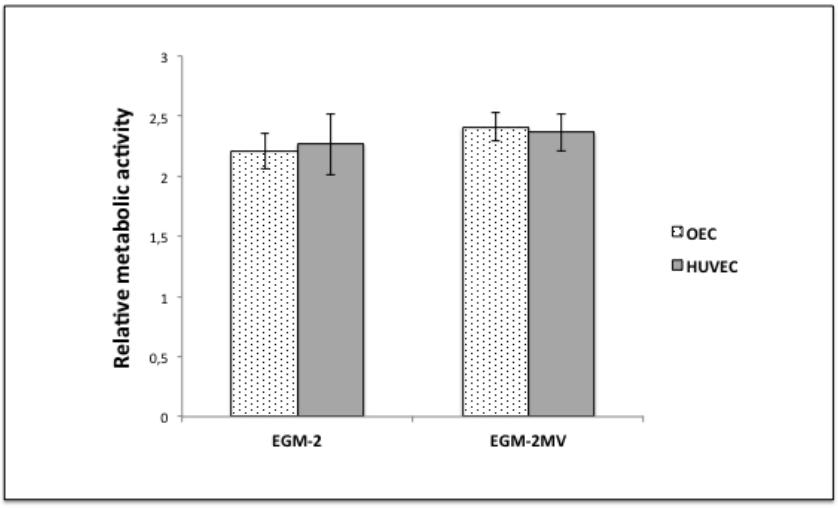


Figure 3.5. EC metabolic activity in response to different culture media. Relative metabolic activity of OECs and HUVECs in response to different culture media by MTS assay, after 24h (n=3).

3.3 Characterization of fibroblasts

For the establishment of a co-culture system, two types of human dermal fibroblasts were used, HFF-1 and HDF. Therefore, fibroblasts were characterized using different markers. The expression of podoplanin (PDPN) was investigated as a marker of dermal fibroblasts and, when monocultures of HFF-1 and HDF were performed using DMEM and EGM-2, it was observed that both types of fibroblasts expressed PDPN after 24h in culture (Fig. 3.6A). Besides, the expression of markers of fibroblast activation, like α-SMA, was also studied. In figure 3.6B, it was observed that both fibroblasts, when cultured alone in EGM-2, exhibited a lower expression of α-SMA after 7 days. However, after 21 days, it was possible to observe an increase in the expression of α-SMA, mainly in the case of HDF (Fig. 3.6B).

In addition, western blot analysis was performed to evaluate the expression of PDPN and α-SMA at the protein level, as well as to investigate the expression of other dermal fibroblast marker, transglutaminase-2 (TG2), in monocultures of HFF-1 and HDF after 7, 14 and 21 days in EGM-2 (Fig. 3.6C). It was verified that both types of fibroblasts expressed all proteins. Nonetheless, HFF-1 exhibited a higher expression of TG2 than that observed for HDF, while HDF expressed higher amounts of PDPN and α-SMA. Furthermore, there was a significant increase in the expression of PDPN between day 7 and day 14, as well as an increase of α-SMA expression from day 7 to day 21 in HDF monocultures.

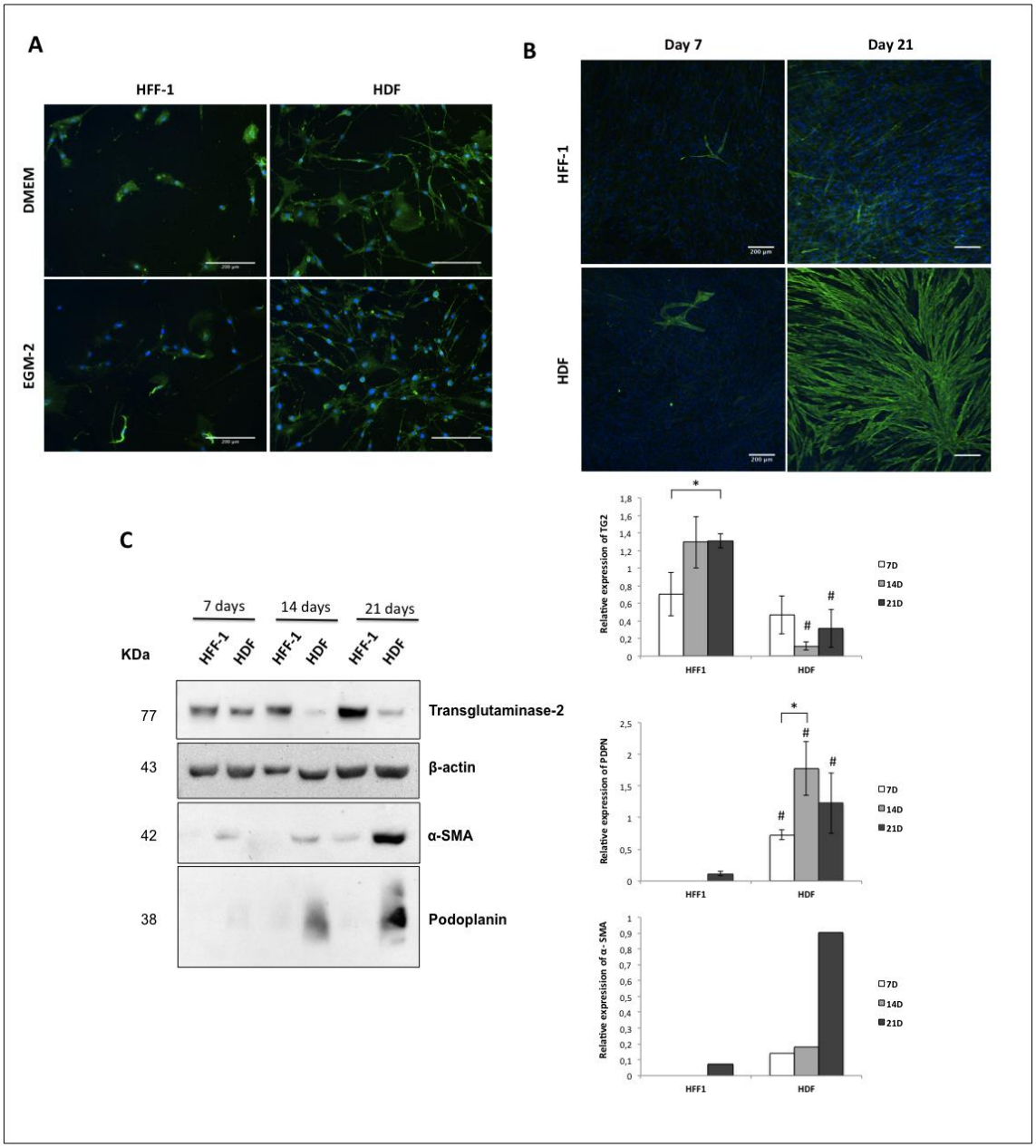


Figure 3.6. Characterization of fibroblasts. (A) Fluorescence microscope images of fibroblasts (HFF-1 and HDF) expressing podoplanin (green) after 24h in culture both in DMEM and in EGM-2 (scale bar, 200 μm). (B) Confocal images of fibroblasts (HFF-1 and HDF) expressing α-SMA (green) after 7 and 21 days in culture in EGM-2 culture medium (scale bar, 200 μm). (C) Western blot and quantitative analysis of transglutaminase-2 (TG2, n=3), podoplanin (PDPN, n=3) and α-SMA (n=1) expressed by fibroblasts after 7, 14 and 21 days. ***** Statistically significant differences ($p<0.05$), between time points. $^{\#}$ Statistically significant differences ($p<0.05$), compared to HFF-1.

3.4 Fibroblasts influenced EC assembly into capillary-like structures

A co-culture system of ECs (HUVECs and OECs) with fibroblasts (HFF-1 and HDF) was established to assess the capacity of different fibroblasts to support the formation of

capillary-like structures by ECs. HUVECs and OECs were cultured alone to serve as controls.

Figure 7 shows the behavior of HUVECs in a monoculture (Fig. 3.7A-C) and in co-culture systems with HFF-1 (Fig. 3.7D-F) and HDF (Fig. 3.7G-I). Here, it was observed that co-culturing HUVECs with HDF resulted in the formation of a capillary-like network after 14 days in culture (Fig. 3.7H), which was maintained at least after 21 days (Fig. 3.7I), with an interconnected network with luminal structures being observed (Fig. 3.8). The same was not observed when HFF-1 were used in the co-culture system, with HUVECs organizing into clusters (Fig. 3.7D-F), although with some protuberant tubular-like structures after 14 (Fig. 3.7E) and 21 (Fig. 3.7F) days. In the control condition, HUVECs stayed in a monolayer over time (Fig. 3.7A-C).

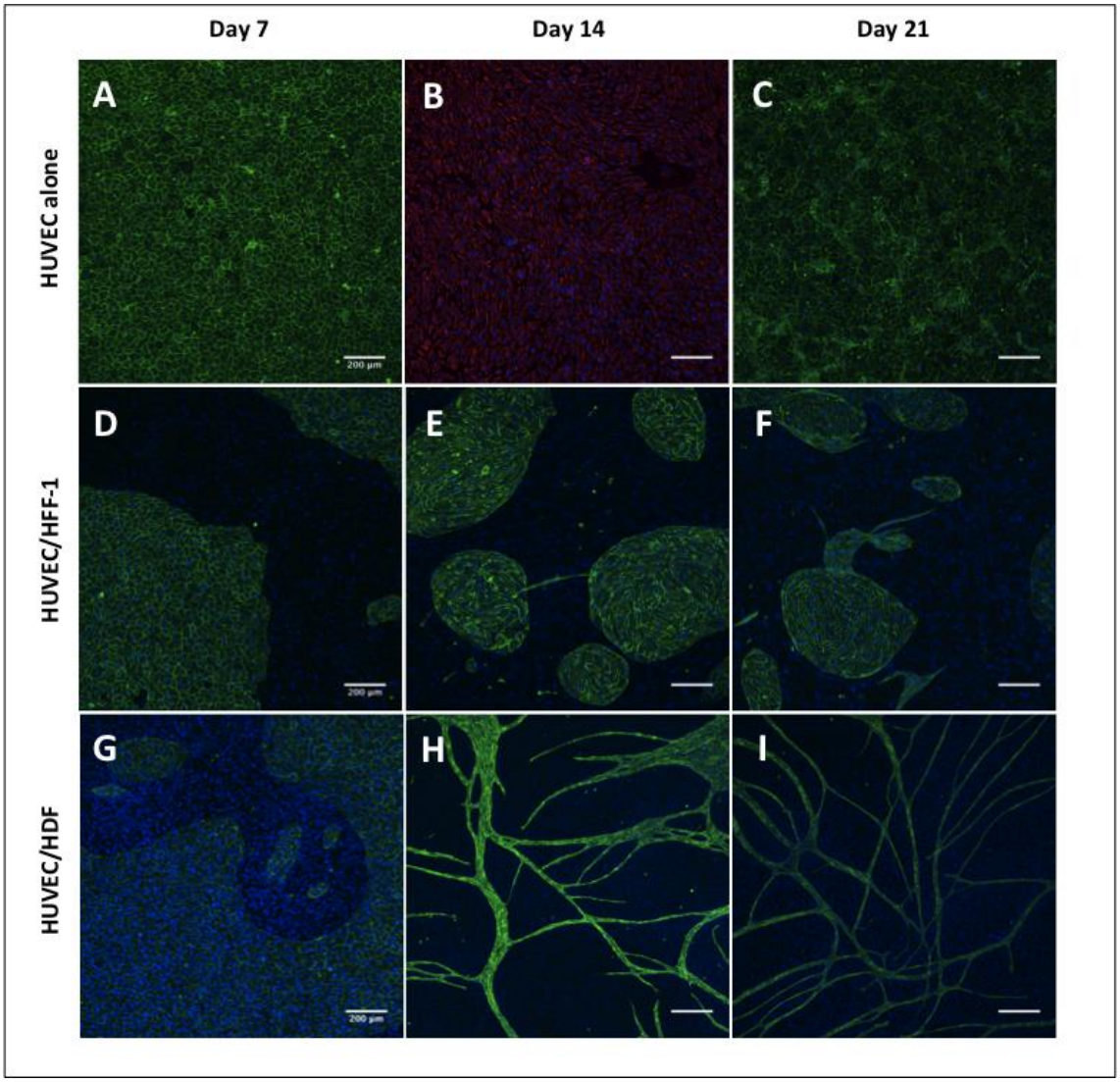


Figure 3.7. Co-cultures of HUVECs with fibroblasts. Confocal images of HUVEC alone (A-C), co-cultures of HUVEC/HFF-1 (D-F) and HUVEC/HDF (G-I) after 7, 14 and 21 days in EGM-2. HUVECs were stained against CD31 (green) or vWF (red) and nuclei were counterstained with DAPI (blue). Scale bar, 200 µm.

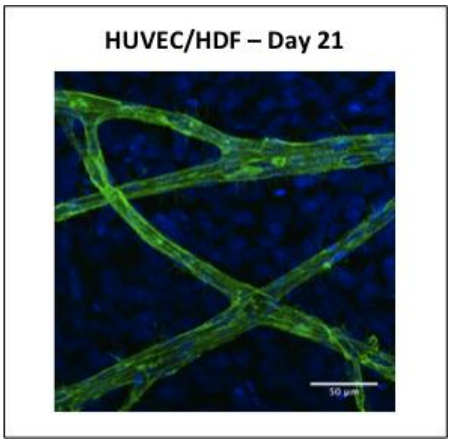


Figure 3.8. Luminal structures in co-cultures of HUVEC/HDF. Confocal image of lumen formation in an interconnected microvascular-like network in co-cultures of HUVEC/HDF after 21 days. HUVECs were stained against CD31 (green) and nuclei were counterstained with DAPI (blue). Scale bar, 50 µm.

OECs were also used in these co-culture systems (Fig. 3.9). When using HFF-1, OECs organized into clusters (Fig. 3.9D-F), whereas when co-cultured with HDF, OECs assembled into capillary-like structures after 14 days (Fig. 3.9H), which were maintained after 21 days in culture (Fig. 3.9I). This is similar to the previously described behavior for HUVECs. When cultured alone, OECs organized into a typical cell monolayer (Fig. 3.9A-C).

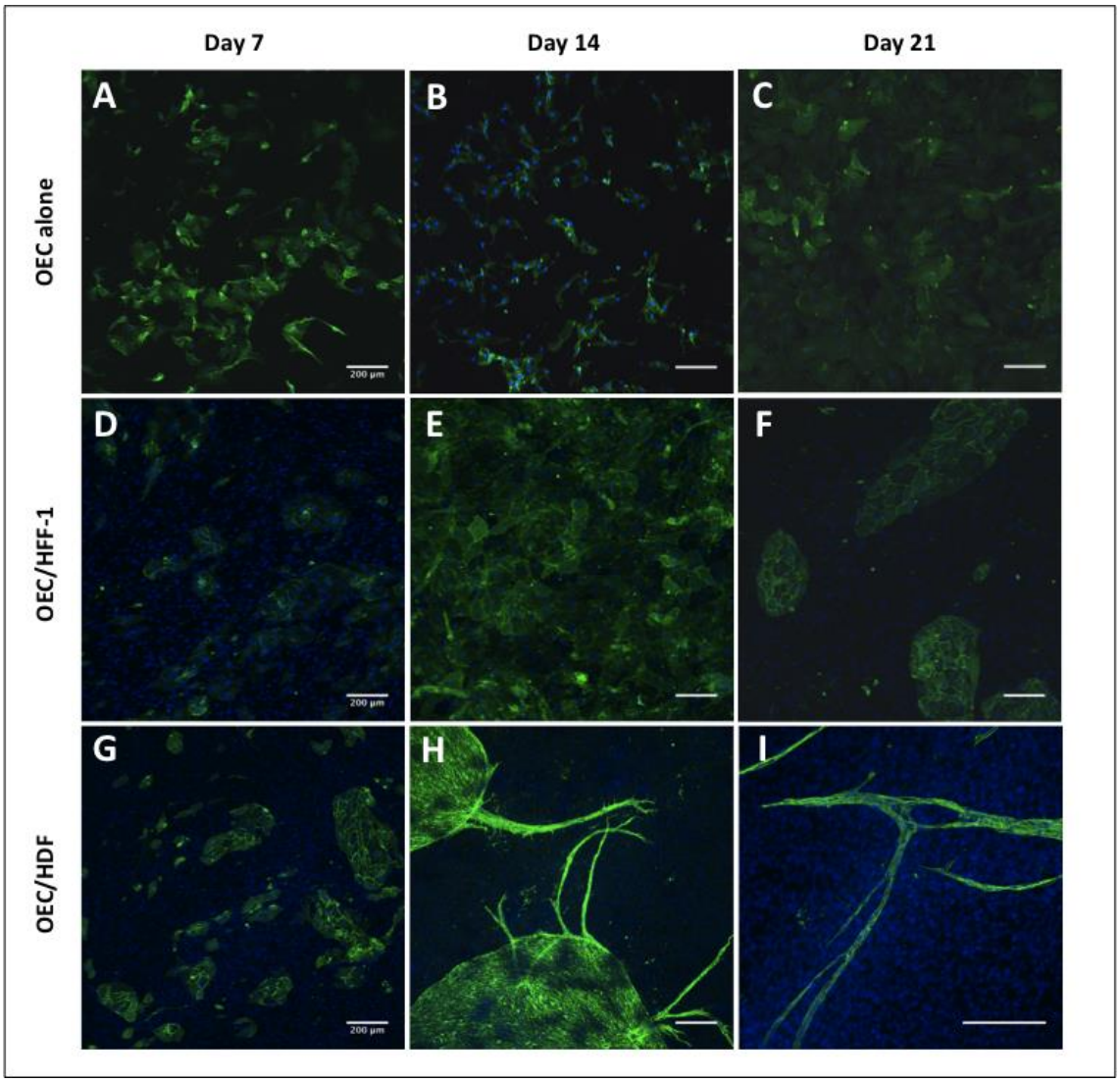


Figure 3.9. Co-cultures of OECs with fibroblasts. Confocal images of OEC alone (A-C), co-cultures of OEC/HFF-1 (D-F) and OEC/HDF (G-I) after 7, 14 and 21 days in EGM-2. OECs were stained against CD31 (green) and nuclei were counterstained with DAPI (blue). Scale bar, 200 µm.

In addition, figure 10 shows a comparison between the ability of HUVECs and OECs to assemble into capillary-like networks in a co-culture system with HDF. Here, it is observed that both types of ECs started organizing into capillaries after 7 days in co-culture with HDF (Fig. 3.10A, 3.10D). HUVEC/HDF co-cultures resulted in the formation of capillaries (Fig. 3.10A-C) in a higher extent than those originated in OEC/HDF co-cultures (Fig. 3.10D-F). Although OECs assembled into structures with a characteristic tubular aspect, HUVECs, on the other hand, were able to organize into a highly branched, interconnected capillary-like network.

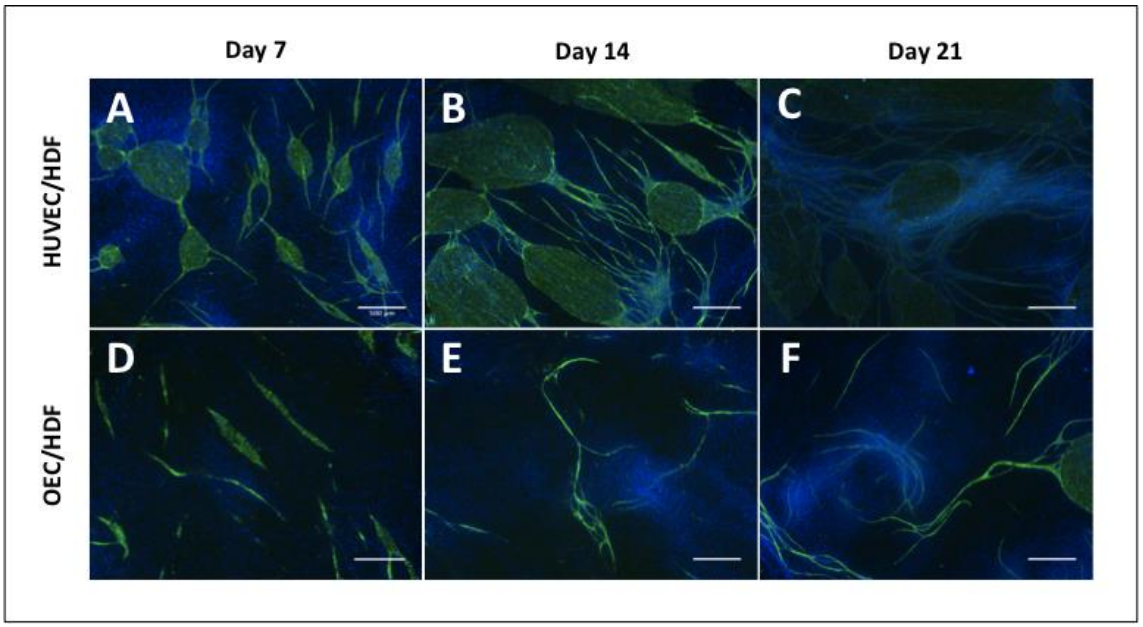


Figure 3.10. Comparison between HUVEC/HDF and OEC/HDF co-cultures. Fluorescence microscope images of co-cultures of HUVEC/HDF (A-C) and OEC/HDF (D-F) after 7, 14 and 21 days. HUVECs and OECs were stained against CD31 (green) and nuclei were counterstained with DAPI (blue). Scale bar, 500 µm.

Therefore, to assess the evolution of this HUVEC-derived capillary-like network over time, parameters including the number of capillary-like structures and branching points, as well as the diameter and length of the capillary-like structures were determined in co-cultures of HUVEC/HDF, after 14 and 21 days (Fig. 3.11). Indeed, there was an increase in the number of capillary-like structures ($p<0.05$, Fig. 3.11A) and an enlargement of these structures ($p<0.05$, Fig. 3.11D) from day 14 to day 21. The number of branching points (Fig. 3.11B) and the diameter of the capillary-like structures (Fig. 3.11 C) remained similar between those two time points.

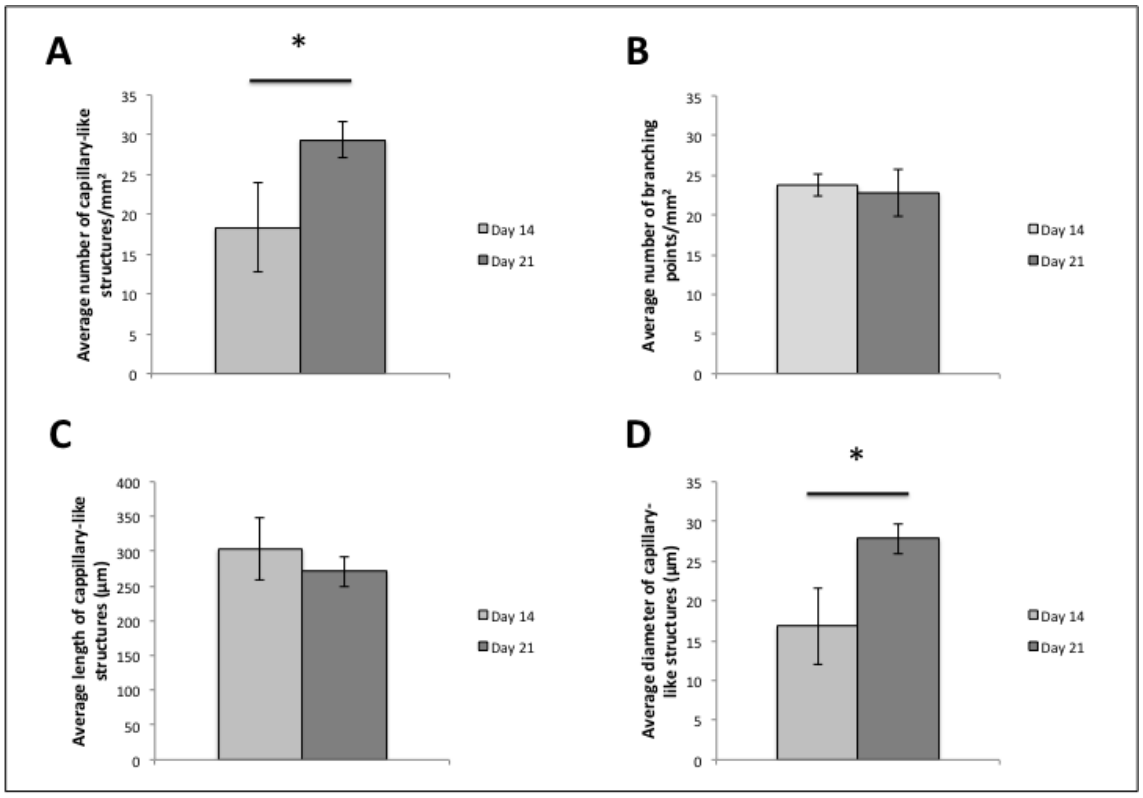


Figure 3.11. Number of capillary-like structures, branching points, length and diameter in HUVEC/HDF co-cultures. Average quantifications of the number of capillary-like structures (A) and branching points/mm^2 (B), the length (C) and the diameter (D) of capillary-like structures formed in co-cultures of HUVEC/HDF after 14 and 21 days. * Statistically significant differences ($p<0.05$, n=6).

3.5 Extracellular matrix production by different cell types

Fibroblasts are the main producers of extracellular matrix components. To evaluate the differences between the ECM produced by distinct types of fibroblasts and endothelial cells, immunostainings against collagen types I and IV, fibronectin and laminin were performed.

It was observed that HUVECs secreted collagen IV, fibronectin and laminin to the extracellular media, but not collagen I (Fig. 3.12). OECs produced the same ECM components at a lower extent and only intracellularly (Fig. 3.12).

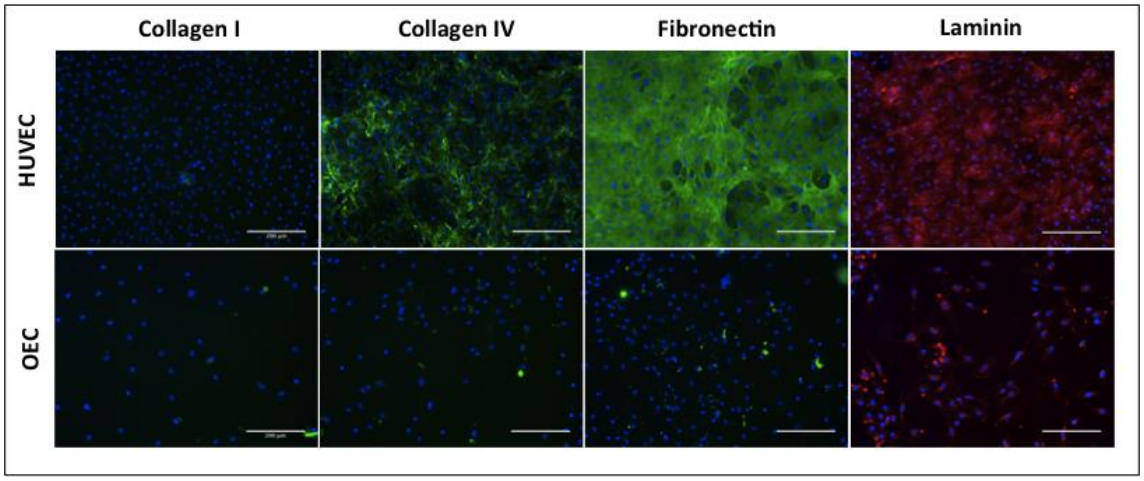


Figure 3.12. ECM components produced by ECs. Fluorescence microscope images of extracellular matrix components – collagen types I (green) and IV (green), fibronectin (green) and laminin (red) – produced by endothelial cells (upper panel – HUVEC and lower panel – OEC) after being cultured for 14 days in EGM-2 culture medium. Nuclei were counterstained with DAPI (blue). Scale bar, 200 µm.

Both types of fibroblasts secreted collagen IV, fibronectin and laminin to the extracellular media, but only HDF secreted collagen I (Fig. 3.13).

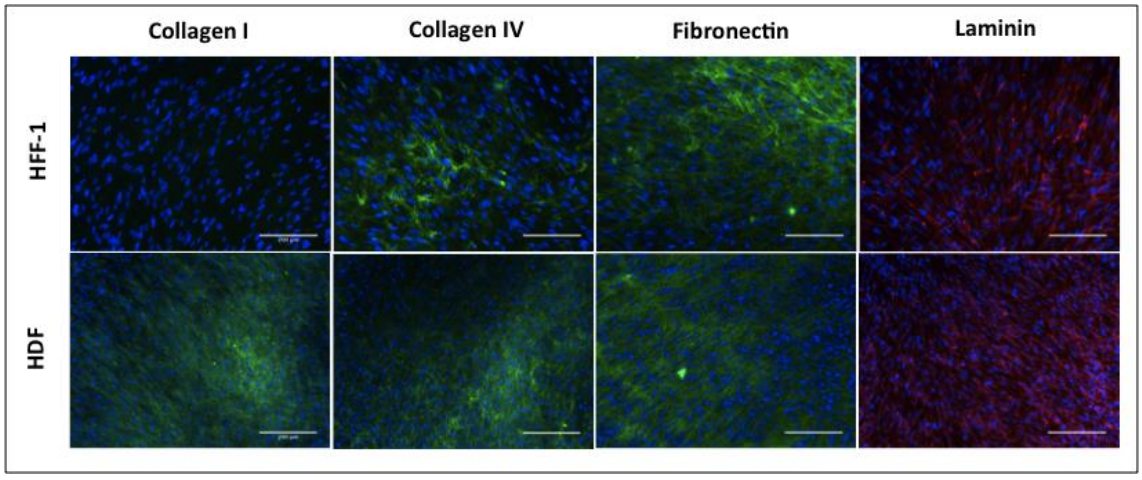


Figure 3.13. ECM components produced by human dermal fibroblasts. Fluorescence microscope images of extracellular matrix components – collagen types I (green) and IV (green), fibronectin (green) and laminin (red) – produced by fibroblasts (upper panel – HFF-1 and lower panel – HDF) after being cultured for 14 days in EGM-2 culture medium. Nuclei were counterstained with DAPI (blue). Scale bar, 200 µm.

For a better understanding about what happens with the ECM during the formation of capillary-like structures, the co-culture of HUVEC/HDF was selected for further investigation of the ECM components. Thus, in co-cultures of HUVEC/HDF, it was observed that all investigated ECM components were present, with collagen types I and IV being mainly expressed where capillary-like structures were present (Fig. 3.14A).

Due to their importance in the constitution of basement membranes, collagen IV and laminin were also investigated by western blot (Fig. 3.14B). Here, it has been verified that collagen IV and laminin were expressed at all time points, although laminin expression decreased from day 7 to day 14.

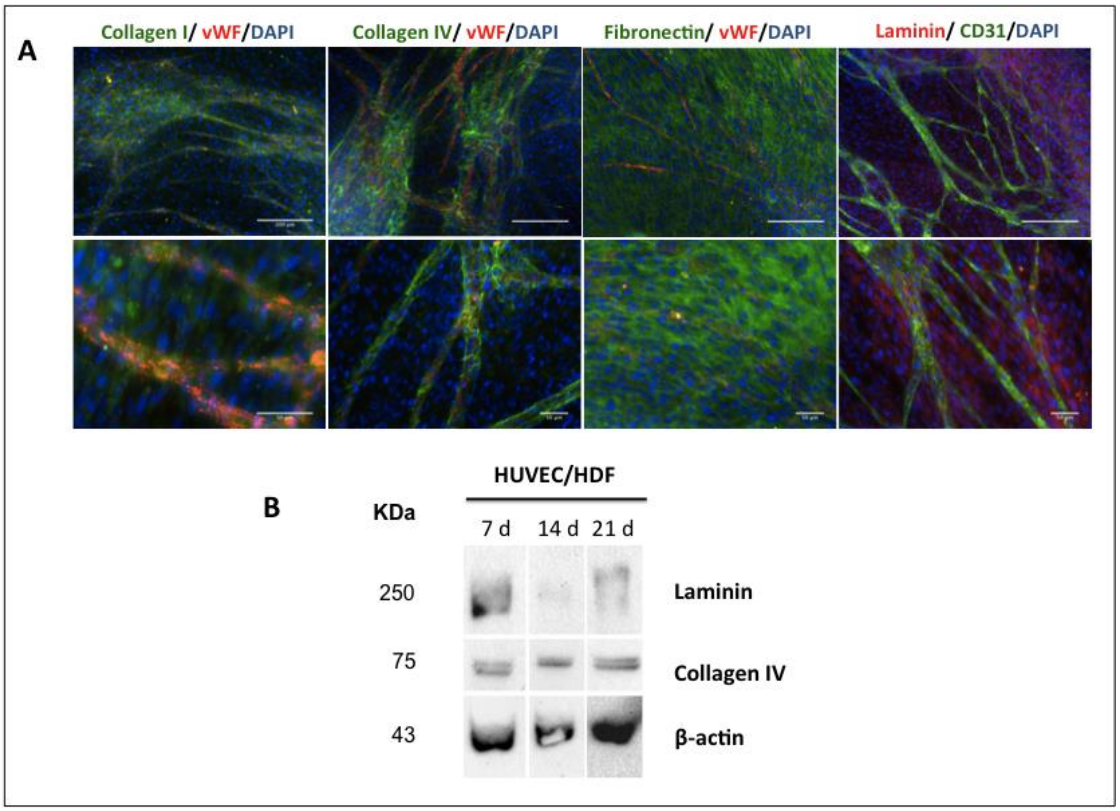


Figure 3.14. ECM in co-cultures of HUVEC/HDF. (A) Fluorescence microscope images of extracellular matrix components – collagen types I (green) and IV (green), fibronectin (green) and laminin (red) –produced in co-culture systems of HUVEC and HDF after 14 days in EGM-2 culture medium. Endothelial cells were stained against vWF (red) or CD31 (green). Nuclei were counterstained with DAPI (blue). Upper panel, scale bar, 200 µm. Lower panel, scale bar, 50 µm. (B) Western blot analysis of collagen IV and laminin expressed in co-cultures of HUVEC/HDF after 7, 14 and 21 days in EGM-2 culture medium.

3.6 EC metabolic activity in response to conditioned media from fibroblasts

In order to investigate whether the factors produced by the two types of fibroblasts affect EC metabolic activity in a paracrine manner, conditioned media from each type of fibroblasts (HFF-1 and HDF) were obtained and used for culturing HUVECs and OECs separately. This effect was compared with the control fresh medium (EBM-2 supplemented with 5% FBS and 1% AB/AM). In what concerns to OECs, only the condition with 100% conditioned medium (CM) from HFF-1 induced a decrease ($p<0.05$ vs control) in the metabolic activity of these cells, in comparison to the control (Fig. 3.15A). Also, when the percentage of CM from HFF-1 is reduced (higher percentage of fresh basal medium), there is a tendency for the metabolic activity of OECs to increase

(Fig. 3.15A). In the case of CM from HDF, no changes in the metabolic activity of OECs (Fig. 3.15B) were observed.

On the other hand, HUVECs exhibited no changes in their metabolic activity when cultured with different percentages of CM from HFF-1 (Fig. 3.15C) and HDF (Fig. 3.15D).

When comparing OECs to HUVECs, cells responded differently to conditions corresponding to 50% conditioned media both from HFF-1 and HDF, with HUVECs exhibiting an increased metabolic activity than that observed for OECs.

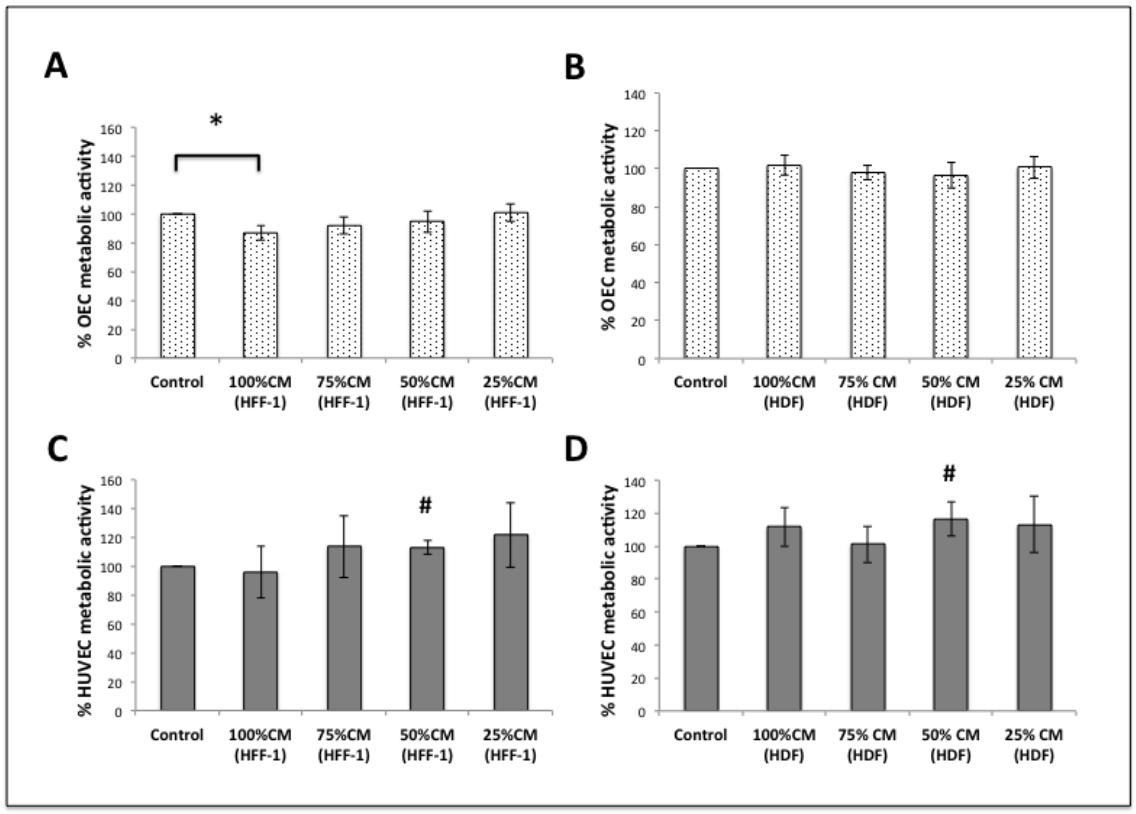


Figure 3.15. EC metabolic activity in response to conditioned media from fibroblasts after 24h. Percent metabolic activity of OECs in response to conditioned media from HFF-1 (A) and HDF (B). Percent metabolic activity of HUVECs in response to conditioned media from HFF-1 (C) and HDF (D). * Statistically significant differences ($p<0.05$ vs control, n=3); # Statistically significant differences – Increased metabolic activity of HUVECs ($p<0.05$ vs OECs, n=3).

3.7 Assembly of ECs into a 3D microcapillary-like network in Matrigel

The Matrigel assay was performed to assess if the presence of fibroblasts (HDF) would influence the ability of ECs (HUVECs or OECs) to assemble into capillary-like structures, as well as to evaluate if these structures could be maintained and stabilized over time. The formation of capillary-like structures can be observed in Fig. 3.16A, with ECs being organized into characteristic polygonal forms after 24h. In Matrigel, co-cultures of HUVEC/HDF and OEC/HDF had a similar behavior to that of monocultures

of HUVECs and OECs, as seen through the number of capillary-like structures (Fig. 3.16B) and branching points (Fig. 3.16C) after 24h. In what concerns to length (Fig. 3.16D) and diameter (Fig. 3.16E) of capillary-like structures, there were no differences between HUVEC or OEC-derived structures, although there was a tendency for HUVECs to originate larger capillary-like structures.

After 48h, the previously formed capillary-like structures started to disaggregate in the case of HUVECs, almost disappearing after 7 days (Fig. 3.16A), with HUVECs adhering to the Matrigel layer. For OECs in monoculture, there seemed to be a higher stability of these structures, which were maintained until day 7 (Fig. 3.16A).

In co-culture conditions, fibroblasts exhibited a high proliferative capacity and migrated to the superficial layer of the Matrigel, instead of staying at the bottom of the culture well. In the case of HUVEC/HDF, capillary-like structures disassembled over time, similarly to the monoculture condition (Fig. 3.16A). However, a different behavior can be observed for OEC/HDF, where capillary-like structures were maintained above layers of migrating fibroblasts.

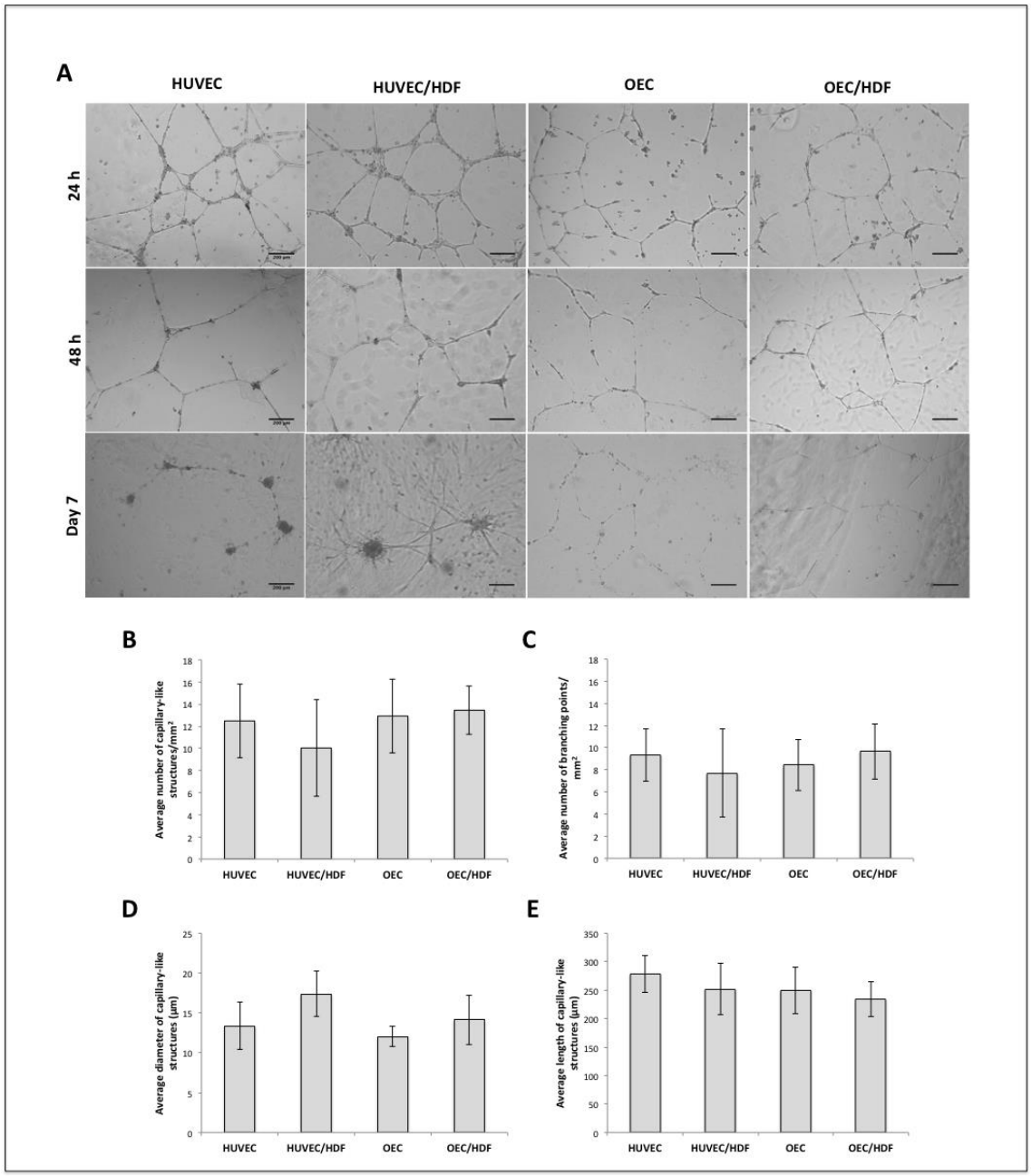


Figure 3.16. 3D Matrigel assay. (A) Optical microscope images of HUVEC, HUVEC/HDF, OEC and OEC/HDF in Matrigel after 24h, 48h and 7 days. Scale bar, 200 µm. (B) Average number of capillary-like structures/mm^2; (C) Average number of branching points/mm^2; (D) Average diameter of capillary-like structures (µm) and (E) Average length of capillary-like structures (µm). B-E were determined after 24h in Matrigel (n=6 for co-cultures; n=4 for monocultures).

CHAPTER IV

DISCUSSION

Over the years, different strategies have been described aiming to achieve the vascularization of an engineered tissue, including cell-based therapies. The discovery of an endothelial progenitor cell (23) has brought new insights into the field of vascular biology and the use of a “true endothelial progenitor cell” could ameliorate strategies based on the transplantation of endothelial cells. Despite the controversy that still exists about terminology and exact origin, OECs seem to fulfill the main requisites for being considered a “true endothelial progenitor cell”: (i) these cells can be easily obtained from circulating blood, constituting an autologous source of ECs; (ii) several endothelial markers are also expressed by OECs; (iii) they present a high expansion potential in culture due to their increased proliferation ability.

In the present study, human OECs have been isolated from umbilical cord blood to analyze their potential contribution for angiogenesis in co-culture systems. These cells exhibited a high proliferation capacity in culture and expressed typical endothelial markers, such as CD31, vWF, VE-cadherin, VEGFR2 and, to a lower extent, CD34, in agreement to past descriptions of this cell population (99). Although CD34 is characteristically expressed by vascular ECs (101), some authors have also reported a low signal for CD34 both in HUVECs and OECs (102), which is in accordance to the fact that $CD34^+$ ECs are enriched for biological functions related to angiogenesis and migration, whereas $CD34^-$ cells are enriched for functions related to proliferation (103). This observation somehow confirms the proliferation state of OECs in monoculture.

In addition, when the angiogenic potential of OECs used here was tested in a Matrigel assay, these cells displayed a good ability for organizing into typical polygonal capillary-like structures at least for 48h, in accordance to what has been described (104).

Since OECs were cultured in EGM-2MV and other works reporting the use of co-culture systems use EGM-2 (22, 98), an MTS assay was performed in order to compare the metabolic activity of ECs (both HUVECs and OECs) in response to these distinct culture media. Here, no differences were observed in EC behavior when cultured either in EGM-2MV or EGM-2. Therefore, EGM-2 culture medium was selected to perform the subsequent angiogenesis assays.

In what concerns to fibroblasts, these cells have traditionally been associated to pathological conditions that involve the development of fibrotic tissue, for instance in cardiac diseases (105, 106). Fibrosis consists on a scarring process that results from fibroblast accumulation, as well as an excessive and disorganized deposition of ECM components (105). However, fibroblasts exhibit pleiotropic functions and are not only matrix-producing cells (106). Therefore, due to their production of biochemical mediators (e.g. growth factors, cytokines and proteases) and their role in the homeostasis of different tissues, fibroblasts have gained increased attention over the years in the field of tissue engineering.

Since fibroblasts are known to be quite different regarding their tissue of origin, one of the purposes of the current study was to compare the behavior of two distinct fibroblast populations – HFF-1 and HDF. HFF-1 are a commercial population of primary neonatal human dermal fibroblasts, whereas HDF constitute a population of primary juvenile dermal fibroblasts. Indeed, human dermal fibroblasts were selected based on the fact that these cells can be easily isolated from skin and, if being autologous to the patient, they carry no risk of rejection or cross-infection (56).

Dermal fibroblasts can be divided into papillary and reticular fibroblasts, and, as previously described by Janson et al., these two cell types might be distinguished by the expression of podoplanin (PDPN) and transglutaminase 2 (TG2), respectively (59).

Different patterns of the expression of PDPN and TG2, as well as of α-SMA were found between HFF-1 and HDF. In fact, HFF-1 expressed higher levels of TG2, whereas PDPN and α-SMA were expressed in higher amounts in HDF.

α-SMA is a known marker of activated fibroblast/myofibroblast differentiation. Transglutaminase-2 belongs to a group of enzymes that catalyze post-translational modification of proteins, through the formation of isopeptide bonds, and is involved in biological processes, such as cell death and differentiation, as well as matrix stabilization (107). Podoplanin is a mucin-like transmembrane glycoprotein and it has been associated to lymphangiogenesis (108). However, there are no studies relating podoplanin to angiogenesis during tissue repair or regeneration.

Considering this data together, it could be hypothesized that HFF-1 would constitute a population of reticular fibroblasts, while HDF would correspond to a population of

papillary fibroblasts. However, care should be taken when extrapolating this conclusion based on the described cell markers, mainly due to the fact that fibroblasts used here were not isolated from the same skin donor site and, consequently, are not so easily comparable. In addition, a recent work has shown that papillary fibroblasts can differentiate into reticular fibroblasts when cultured over several passages (109). Also, a noteworthy aspect is that the same authors have attributed a higher expression of α-SMA to reticular fibroblasts (59, 109), whereas in the populations described here high levels of this marker were found to be expressed by HDF.

Consequently, both types of fibroblasts were used to examine their capacity to influence the formation of capillary-like structures either by macrovascular and progenitor ECs (HUVECs and OECs, respectively). HDF were found to induce to a high extent the formation of capillary-like structures, while HFF-1 failed to promote EC organization into tubular structures. Indeed, HDF had the ability to promote the assembly of HUVECs into a complex interconnected capillary-like network after 14 days and to support the maintenance of this network at least until day 21. In this culture system, the support of HDF resulted in a higher number and in an enlargement of HUVEC-derived capillaries caliber between days 14 and 21. Identical to its influence in macrovascular ECs, HDF also induced OECs to assemble into capillary-like structures for 21 days.

This difference between the ability of HDF and HFF-1 to induce the formation of capillaries *in vitro* might be due to the fact that HDF expressed markers of papillary fibroblasts (PDPN), while HFF-1 expressed markers of reticular fibroblasts (TG2). As previously mentioned, it is known that, contrary to papillary fibroblasts, reticular fibroblasts seem to have a lower ability to support the formation of tubular-like structures *in vitro* (60). Indeed, TG2 has been described as a partner of endostatin, an anti-angiogenic peptide present in the ECM close to ECs (110).

Although ECs are a rich source of TG2 (111), the presence of TG2 produced by the HFF-1 might be one of the reasons for the observed inhibition of EC assembly into capillary-like structures, as it has already been described that the addition of exogenous TG2 blocks angiogenesis *in vitro* (112). In the present work, HUVECs were found to be expressing TG2 when cultured alone for 7, 14 and 21 days (data not shown). However, expression of TG2 was not verified when formation of capillary-like structures occurred, namely in co-cultures of HUVEC/HDF (data not shown), which corroborates the fact that the presence of TG2 produced by fibroblasts (HFF-1) might be an inhibitor of the formation of capillary-like structures.

Nevertheless, previous studies from the same team have shown that when HFF-1 were entrapped in an artificial extracellular matrix, such as modified alginate with RGD peptidic sequence (arginine-glycine-aspartic acid, RGD), the capillary-like structures

were maintained during 5 days (94). In addition, when HFF-1 were used in a model of Matrigel plug implantation in mice, these cells induced the ingrowth of blood vessels from the host vasculature into the plug (93). This raises the question about to what extent can a biomaterial modulate the crosstalk between cells in direct contact. Thus, more studies at the molecular level will be useful to help clarifying this issue.

Another hypothesis to explain the distinct behavior of ECs in the described co-culture systems is based on distinct profiles of soluble factors or ECM components being produced by HFF-1 or HDF. Thus, HUVECs and OECs were cultured with conditioned media obtained from both types of fibroblasts, in order to investigate if there was any difference on EC metabolic activity in response to factors produced either by HFF-1 or HDF. Using MTS assay, it was observed that there were no differences in EC response to HDF-conditioned medium. However, conditioned medium from HFF-1 significantly reduced OEC metabolic activity, when these cells were cultured without the addition of fresh medium (100% CM from HFF-1). Indeed, there was a tendency for OEC metabolic activity to increase, when cultured with decreasing percentages of CM from HFF-1. Significant differences were also obtained when comparing the response between HUVECs and OECs to the conditions that had 50% CM both from HFF-1 and HDF (50% fresh medium).

Since no exogenous growth factors were added to the medium and OECs, as microvascular progenitor cells, are more sensitive to the lack of factors, it can be hypothesized that the distinct ability of both fibroblasts to support the formation of capillaries *in vitro* may not result from distinct patterns in terms of the secretion of soluble factors.

Sorrell et al. 2008 seeded together papillary and reticular fibroblasts in a dish and observed a higher formation of capillary-like structures in the area where papillary fibroblasts were present (60). This suggested that either ECM molecules or matrix-bound molecules would be critical for the formation of capillary-like structures; otherwise the release of factors to the medium would have been sufficient to obtain an homogenous formation of tubular structures by ECs.

Consequently, all cell types used in the present study were further characterized in terms of ECM components secretion, including collagen types I and IV, fibronectin and laminin.

One striking difference between HUVECs and OECs is their release of ECM components. Accordingly, HUVECs were found to secrete collagen type IV, fibronectin and laminin to the extracellular media, whereas in OECs these proteins were only detected intracellularly. HUVECs have been described to produce collagen IV, fibronectin and laminin only when cultured under hypoxic conditions (113). However, this

latter work used shorter time points (1 and 4 days in culture). In the current work, it has been shown that macrovascular ECs, namely HUVECs, are also ECM producers not only after 14 days as described, but also at day 7 and 21 (data not shown).

In what concerns to fibroblasts, HDF were able to secrete all the investigated ECM components, primarily collagen I, which is lacking in HUVECs, OECs and HFF-1. Therefore, the role of HDF as stimulators of the formation of vascular structures probably depends on the secretion of these components.

As the production and deposition of ECM components result in the assembly of a 3D-like matrix and since cells are naturally embedded in a 3D microenvironment, a 3D Matrigel assay was performed to evaluate the behavior of a co-culture system of ECs (HUVECs and OECs) with HDF. After 24 and 48h, it was observed that HUVECs and OECs had a similar behavior when co-cultured with HDF, with capillary-like structures being of a larger caliber in co-culture conditions, compared to ECs alone. Nevertheless, after 7 days, capillary-like structures previously formed in HUVEC monocultures appeared to be disaggregating and almost disappearing in co-cultures of HUVEC/HDF. In the case of progenitor cells, capillary-like structures were still observed both in OEC monocultures and in co-cultures of OEC/HDF after 7 days. This result suggests that using OECs in a 3D matrix might be a better strategy to promote vascularization.

However, care should be taken when extrapolating conclusions from these results. In the present work, two distinct systems were compared: one in 3D without the addition of an external ECM (co-culture) and another in 3D with the addition of an external ECM (matrigel) at the beginning of the assay. Despite being a common *in vitro* 3D assay to evaluate tubule formation, GFR-Matrigel results in the formation of short and relatively homogeneous capillary-like structures (114). On the other hand, co-cultures of ECs with fibroblasts originate a highly heterogeneous network of interconnected capillary-like structures, resembling *in vivo* capillary organization (114). Here, another relevant aspect to take into consideration is the time course over which both assays were carried out. Longer time points used for performing the 3D co-culture system (14 and 21 days) could not be used for Matrigel assay. Indeed, Matrigel would be degraded if extensive time points were used, due to the presence of proteases secreted by migrating cells (115).

Altogether, these findings suggest that HDF is a preferential cell source for enhancing vascularization, both in HUVECs and OECs. Given the already described advantages of OECs, these findings open a new field of research regarding the use of specific fibroblast populations co-cultured with OECs, as efficient partners for vascular development with tissue regeneration purposes. Finally, *in vivo* implantation of OECs together with HDF would further elucidate their role in vascular tissue engineering.

CHAPTER V

CONCLUSIONS AND PERSPECTIVES

Cellular strategies constitute a promising way to achieve vascularization of tissue engineering constructs avoiding the undesirable side effects of growth factor, cytokine, hormone or other bioactive molecules delivery.

The interactions between endothelial cells and other cell types seem to constitute a bi-directional system. Co-culture systems are a useful instrument providing new insights into the molecular mechanisms underlying vascularization, since they are capable of mimicking physiological processes. Cell-cell contacts correspond to a precise representation of *in vivo* tissues, enhancing cell-specific activities. In general, microvessel-like structures are formed in these co-culture systems.

Endothelial progenitor cells have brought new prospects and are currently being investigated as a potential cell source for therapeutic applications requiring vascularization. OECs in particular are considered an especially interesting source of autologous endothelial cells for tissue engineering owing to their high proliferative capacity.

The present work focused on the *in vitro* formation of capillary-like structures, aiming to understand the importance of cell communication in a co-culture system.

On the one hand, monocultures of OECs and HUVECs have been reported to behave similarly in terms of angiogenic activity. On the other hand, different results were obtained from co-cultures, with OECs exhibiting an enhanced vessel formation when other cell types were present. These results suggest that further investigation should be performed at the molecular level to gain insight into such differences. Standard protocols

should be established in order to overcome the present limitations, as well as to allow for a more accurate definition of the functional potential of OECs, helping to understand their regenerative potential for clinical applications.

In vivo formation of perfused microvessels by ECs appears to be dependent on their communication with other vascular cell types, like pericytes, SMCs and fibroblasts, as well as with other specialized cell types, such as osteoblasts, MSCs, and others, with a beneficial effect for vascular structures formation and stabilization.

However, key issues related with vascularization remain unanswered, including: (i) the lack of uniform cellular definitions, since phenotypical characterization is still controversial; (ii) the inadequate functional characterization; (iii) origin of the true source of EPCs and whether there exists a relationship with the hematopoietic lineage; (iv) preferable cell type for a therapeutical application; (v) most efficient strategy to apply OECs for complex vascularized tissue construct formation; and (vi) the need to establish standardized protocols for OECs isolation and expansion, in order to improve the comparison between different studies. Furthermore, it is also of major importance to determine the temporal window and the sequence of events leading to the formation of a functional microvasculature.

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